Genome scale analysis of the role of superantigens in *Staphylococcus aureus* disease pathogenesis

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Declaration

I declare that I have composed this thesis, that the work described here is my own, and has not been submitted for any other degree or professional qualification.

Gillian Wilson

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List of commonly used abbreviations

Abbreviation	Full description
α	alpha
agr	accessory gene regulator
AIP	auto inducing peptide
APC	antigen presenting cell
APS	ammonium persulphate
β	beta
BLAST	basic local alignment search tool
bovVβ	bovine variable beta
bp	base pair
BSA	bovine serum albumin
BHI	brain heart infusion
°C	degrees Celsius
CA-MRSA	community-acquired methicillin resistant *Staphylococcus aureus*
CC	clonal complex
CDC	Centers for Disease Control
cDNA	complementary deoxyribonucleic acid
CFSE	caboxyflourescein succinamidyl ester
CFU	colony forming units
CO_2	carbon dioxide
ConA	concanavalin A
cpm	counts per minute
C_T	threshold cycle
CWA	cell wall-associated
d	days
dATP	2'-deoxyadenosine triphosphate
dCTP	2'-deoxycytidine triphosphate
ddH_2O	double distilled water
dGTP	2'-deoxyguanosine triphosphate
DMSO	dimethyl sulphoxide

Abbreviation	Full description
dTTP	2'-deoxythymidine triphosphate
dNTP	mixture of dATP, dCTP, dGTP and dTTP
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
EDTA	ethylene-diamine-tetra-acetic acid
egc	enterotoxin gene cluster
ET	electrophoretic type
FACS	fluorescence-activated cell sorting
FCS	foetal calf serum
x g	force of gravity
γ	gamma
g	grams
gDNA	genomic DNA
h	hours
HBSS	hanks buffered saline solution
HCl	hydrochloric acid
HGT	horizontal gene transfer
HLA	human leukocyte antigen
^{3}H	hydrogen radioactive isotope 3 (tritium)
humVβ	human variable beta
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IMGT	Immunogenetics Database
IMI	intra-mammary infection
Ig	immunoglobulin
IPTG	β-D-1-thiogalactpyranoside
kDa	kilo-Dalton
kV	kilo-volts
L	litre
LB	Luria-Bertani broth

Abbreviation	Full description
kb	kilo-base
MEGA	Molecular Evolutionary Genetics Analysis
mM	milli-molar
MGE	mobile genetic element
MHC	Major histocompatability complex
MHC class I	Major histocompatability complex class I
MHC class II	Major histocompatability complex class II
MLEE	multi locus enzyme electrophoresis
MLST	multi locus sequence type
min	minutes
µg	micro-grams
µF	micro-Faraday
ml	milli-litres
µl	micro-litres
µCi	micro-curie
mRNA	messenger ribonucleic acid
MRSA	methicillin-resistant *Staphylococcus aureus*
MSSA	methicillin-susceptible *Staphylococcus aureus*
MWCO	molecular weight cut off
NCBI	National Centre for Biotechnology Information
ng	nano-grams
nm	nano-metre
nM	nano-molar
ND	not done
NK	not known
NTC	no template control
OD	optical density
PBL	peripheral blood lymphocyte

Abbreviation	Full description
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDB	protein data bank
pmol	pico-molar
PSG	penicillin, streptomycin, L-glutamine
PVL	Panton-Valentin leukocidin
qRT-PCR	quantitative reverse transcriptase-polymerase chain reaction
RBC	red blood cells
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcriptase-polymerase chain reaction
RT	reverse transcriptase
s	second
SAg	superantigen
SaPIbov	bovine staphylococcal pathogenicity island
SCC	somatic cell counts
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SE	staphylococcal enterotoxin
SEl	staphylococcal enterotoxin like
ST	sequence type
TAE	tris-acetate ethylene-diamine-tetra-acetic acid
TBE	Tris-borate/EDTA
TCR	T-cell receptor
TCS	Two component system

Abbreviation	Full description
TE	Tris- ethylene-diamine-tetra-acetic acid
TEMED	tetramethylethylinediamine
Th1	T helper 1
TNF	Tumor necrosis factor
TRBV	T-cell receptor beta variable
T_{regs}	Regulatory T-cell
TSA	Tryptone soya agar
TSB	Tryptone soya broth
TSS	Toxic shock syndrome
TSST-1	Toxic shock syndrome toxin-1
U	units
UV	ultraviolet
V	volts
v/v	volume for volume
WT	wild type
w/v	weight for volume

Abstract

Staphylococcus aureus produces a family of at least 21 distinct superantigens (SAgs) which include staphylococcal enterotoxins (SEs), staphylococcal enterotoxin-like toxins (SEls), and toxic shock syndrome toxin-1 (TSST-1), and contribute to disease pathogenesis via modulation of the host immune response. Specific SAgs have been shown to cause toxinoses such as staphylococcal food poisoning and toxic shock syndrome, and have been implicated in immunological disorders such as rheumatoid arthritis, psoriasis, and Kawasaki syndrome. However the role of SAgs in disease pathogenesis, in general, is poorly understood.

S. aureus is a common cause of bovine mastitis. Analysis of the genome sequence of the bovine strain RF122 revealed genes encoding bovine variants of characterized SAgs, TSST-1, SElL, SEC, SEG, SEI, SElU, SElO, SElN and a truncated form of SElM. In addition we identified 3 genes with sequence homology to characterized SAgs, which are predicted to encode novel SAgs, SElX, SElY and SElZ. Expression of all 11 predicted SAg genes was detected *in vitro*, including several with growth phase-dependent expression.

Characterization of a novel SAg, SElX which is encoded in the core genome of 94% of phylogenetically diverse *S. aureus* strains from human and animal infections was carried out. In addition to its superantigenic properties, SElX has a unique predicted structure characterized by a truncated SAg B domain. At least 14 different alleles of the *selx* gene were identified among the common human and animal pathogenic clones, and evidence for assortive recombination of *selx* alleles between distinct clonal lineages was discovered. SElX was expressed by representative human, bovine and ovine strains *in vitro,* in a growth phase dependent manner, and during human, bovine and ovine infections, consistent with a broad role in the pathogenesis of different *S. aureus* diseases in multiple hosts. SElX produced by bovine- and ovine-specialized *S. aureus* strains had 10-fold greater mitogenic activity and a distinct Vβ activation profile for bovine lymphocytes compared to SElX made by the human strain USA300 indicating functional diversification of *selx* alleles from different hosts. This is the first description of a core-genome encoded

SAg of *S. aureus*. The discovery that the great majority of *S. aureus* clinical isolates have superantigenic capacity has important implications for our understanding of staphylococcal disease pathogenesis.

To investigate the role of SAgs in disease pathogenesis, a SAg-deficient strain of *S. aureus*, RF122-8 was constructed by sequential allele replacement. RF122-encoded SAg genes were cloned into the pALC2073 plasmid, which has an inducible promoter allowing controlled expression in the SAg-deficient strain RF122-8. These constructs allowed us to determine that TSST-1_{bov}, SElL_{bov}, SEC_{bov}, SElN_{bov}, SEI_{bov} and novel SAgs, SElX_{bov} and SElY_{bov} were mitogenic for bovine T-cells, and stimulated T-cell receptor β variable (TRBV) sub-family-specific activation. Preliminary experimental intra-mammary infections of dairy cows revealed that clinical symptoms were similar during infection with wild type RF122 and SAg-deficient strains, including high somatic cell counts (6 Log^{SCC}), and elevated body temperature (106 °F). However a higher infectious dose was required to establish infection with the SAg-deficient strain RF122-8 in comparison to the wild type, RF122 indicating an attenuation of virulence.

Overall, these data provide broad new insights into the importance of SAgs in staphylococcal disease pathogenesis.

Chapter 1

Introduction

1.1 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive bacterium which was first identified in association with a surgical wound infection by Alexander Ogston in 1882 (Ogston, 1882). *S. aureus* is a facultative anaerobe, able to exist both in the environment and as an external commensal of humans and other animals (Lowy, 1998). The organism is frequently isolated in the anterior nares, female genital tract and skin surface of humans, and is found in the nostrils, rectum and teat skin of ruminants (Fitzgerald, 2008, Lowy, 1998). Studies have identified three nasal carriage patterns within adult human populations, of which 20% are persistent *S. aureus* carriers, 60% are intermittent and 20% are persistent non-carriers (Peacock *et al.*, 2001, Gould & Mc, 1954, Kluytmans *et al.*, 1997). Clinical infections occur if skin or mucosal barriers are breached (Ferens & Bohach, 2000, Lowy, 1998), and in the hospital setting, carriers are at an increased risk of *S. aureus* infection, caused in 80% of cases by the colonising strain (Wertheim *et al.*, 2004). In addition, colonisation is known to be a risk factor in community-acquired infections (von Eiff *et al.*, 2001).

S. aureus is a major human pathogen, responsible for an array of different diseases including, septicaemia, endocarditis, osteomyelitis, localised abscesses and toxigenic illnesses such as toxic shock syndrome (TSS), scalded skin syndrome (SSS) and necrotising pneumonia (Lowy, 1998). *S. aureus* is a major cause of nosocomial infection, and the increased spread of the organism has been accompanied by an increase in antibiotic resistance. In Europe, Japan and the USA, it has been reported that between 40% to 60% of hospital acquired *S. aureus* infections are resistant to methicillin (Tiemersma 2004, Grundmann *et al.*, 2006). Methicillin-resistant *S. aureus* (MRSA) strains were first identified in the 1960s, however it was not until the late 1980s that pandemic spread of MRSA occurred (Grundmann *et al.*, 2006). Although MRSA infections can be successfully treated with vancomycin, the emergence of vancomycin resistance has been reported (Chang *et al.*, 2003, Grundmann *et al.*, 2006). In addition, increasingly virulent CA-MRSA strains have emerged in the community causing severe skin and soft-tissue infections, which are characterised by carriage of type IV or V staphylococcal cassette chromosome (SCCmec), and are often associated with the production of

Panton-Valentin leukocidin (PVL) toxin (Morgan, 2007, Lina *et al.*, 1999). However the role of PVL in CA-MRSA is controversial as not all strains express the toxin (Said-Salim *et al.*, 2005), and contrasting results have been obtained in animal models (Wardenburg *et al.*, 2008, Labandeira-Rey *et al.*, 2007, Voyich *et al.*, 2006). The emergence of highly-virulent and antibiotic-resistant strains highlights the need to understand the pathogenesis of this successful and rapidly-evolving bacterium.

1.2 Bovine mastitis

S. aureus can infect a diverse range of host species causing diseases such as staphylococcosis in rabbits, pyaemia in sheep, chondronecrosis, septicaemia and dermatitis in chickens, pneumonia and osteomyelitis in turkeys and mastitis in cattle (McNamee *et al.*, 1999, Hermans *et al.*, 2003, Takeuchi *et al.*, 2002, Ferens & Bohach, 2000, Webster & Mitchell, 1989, Miles *et al.*, 1992). *S. aureus* is one of the most common causes of bovine mastitis, an infection of the milk-secreting tissue of the udder, which represents a huge economic problem for the dairy industry worldwide (Miles et al., 1992, Barkema *et al.*, 2006). Economic loss in the United States due to reduced quantity and quality of milk, and increased veterinary and labour costs associated with mastitis is estimated to be over $2 billion annually (Wells *et al.*, 1998). Symptoms observed in dairy cows include inflammation of the udder, flakes or clots in milk, fever, loss of appetite, dehydration and death (Schroeder, 1997). Subclinical infection is the most common form of disease, where milk production is lowered by 10% to 25% (Sutra & Poutrel, 1994). Antibiotic treatment is often not curative, instead converting a clinical infection to a latent or subclinical infection, and has led to concern that milk containing antibiotic products may be contributing to the growing problem of antibiotic resistance (David, 1999). *S. aureus* is known to adhere to keratinized cells of the teat canal, and is internalised by mammary epithelial cells (Bayles *et al.*, 1998). Bovine mastitis caused by *S. aureus* infection is typically chronic in nature and the host is often unable to completely eliminate the organism (Ferens & Bohach, 2000). The factors responsible for persistence in the host are not well understood.

1.3 Population genetics of *S. aureus*

The highly clonal population structure of *S. aureus* has been demonstrated by multi locus enzyme electrophoresis (MLEE) and multi locus sequence typing (MLST) studies (Feil *et al.*, 2003, Musser *et al.*, 1990). There are at least 10 major clonal complexes (CC) amongst human strains, of which CC5, CC8, CC22, CC30, CC45 and hybrid lineage CC239 have acquired *mecA* and become widespread in the hospital environment. In addition, strains belonging to CC1, CC8, CC30, CC59 and CC80 which have acquired *mecA* have become prevalent in the community, causing CA-MRSA infections (McCarthy & Lindsay, 2010). Companion animals are usually colonised and infected by common human lineages (Loeffler *et al.*, 2005). Unique phenotypes of animal strains were identified in the 1930s, which led to the identification of ecological variants (ecovars) specific for different host species (Devriese, 1984). A number of population genetic studies have revealed that strains belonging to specific CCs including, CC97, CC71 and CC151 are associated mainly with ruminants and rarely with humans, suggesting they are host specific (Jorgensen *et al.*, 2005, Fitzgerald, 1997, Kapur *et al.*, 1995, Smyth *et al.*, 2009, Guinane *et al.*, 2010). In cattle it has been determined that a few specialised clones of *S. aureus* with broad geographic distribution are responsible for the majority of cases of bovine mastitis (Smyth *et al*., 2009, Fitzgerald *et al.*, 1997, Guinane *et al*., 2010). In particular, CC151 is a bovine-specialised clone which has not been detected amongst humans (Guinane *et al.*, 2008).

Bacterial evolution occurs in part through horizontal gene transfer (HGT) mechanisms such as transformation, transduction and conjugation. Some Gram-positive species such as the streptococci are naturally transformable and are able to take up free DNA from their environment, leading to freely recombining populations. In contrast, *S. aureus* is not naturally transformable and point mutations are known to exceed recombination events (Witte, 2006). Mobile genetic elements (MGE) such as pathogenicity islands, plasmids, phage, transposons and insertion sequences, are usually passed on to daughter cells by vertical transmission leading to a strong association with clonal lineage, or horizontally transferred which allows dissemination between lineages (Lindsay & Holden, 2006). Absence of

MGE from particular lineages can be due to restrictions on horizontal transfer (Kuroda *et al.*, 2001).

1.4 Comparative Genomics

Substantial genomic heterogeneity exists between *S. aureus* strains. An understanding of which combinations of genes are responsible for the success of dominant clonal lineages of *S. aureus* is very important. DNA microarray analysis of diverse strains showed that about 78% of genes were common amongst all strains, forming a core genome (Fitzgerald *et al.*, 2001b, Lindsay & Holden, 2006). The core genome is comprised of genes essential for growth and survival, including some genes encoding virulence factors such as cell wall-associated (CWA) proteins, certain toxins, and the polysaccharide capsule (Lindsay & Holden, 2006). The remaining 22% of the genome is composed of strain-specific accessory genetic material including genes involved in colonisation of specific host or environmental niches. The accessory genome includes 18 large chromosomal regions of difference (RDs) including MGEs, and core variable regions, comprising of lineage-specific genes (Fitzgerald *et al.*, 2001b). The wide distribution of MGEs in strains of divergent clonal lineages which do not share a common ancestor indicates that HGT has been central to the evolution of pathogenic *S. aureus* strains (Fitzgerald et al., 2001b). For example, the genome sequences of an MRSA isolate, MRSA252, and a methicillin sensitive *S. aureus* isolate, MSSA476 were compared, and the isolates differed considerably in their accessory elements (Holden *et al.*, 2004). MRSA252 was found to be particularly genetically diverse with 6% of the genome novel in comparison to other published genomes (Holden *et al.*, 2004). Recently, the genome sequences of *S. aureus* isolates from bovine, ovine and avian hosts have been determined and comparative genomic analysis has revealed molecular evidence for staphylococcal adaptation to different host species, including gene decay and acquisition of genes encoding proteins involved in host-pathogen interactions (Herron-Olson *et al.*, 2007, Lowder *et al.*, 2009, Guinane *et al.,* 2010*)*.

1.5 Pathogenesis of *S. aureus*

S. aureus pathogenesis is multi-faceted, and mediated by a vast range of virulence factors including secreted and CWA proteins. The complement of virulence factors allows the organism to evade the host immune system, adhere to extracellular matrix molecules, enter host cells and spread within the tissues. Most *S. aureus* strains produce a polysaccharide capsule which interferes with phagocytosis and allows the bacteria to survive in the bloodstream (O'Riordan & Lee, 2004). There are 11 capsular serotypes of which type 5, 8 and 336 are most common among clinical isolates (Roghmann *et al.*, 2005, O'Riordan & Lee, 2004).

CWA proteins are particularly important as they allow the organism to adhere to host tissue components, bind proteins in blood, and resist phagocytosis (Joh *et al.*, 1999, Skaar & Schneewind, 2004, Foster & Hook, 1998). In addition, several CWA proteins promote iron uptake and contribute to biofilm formation (Lowy, 1998, Otto, 2008). Well characterised CWA proteins include Protein A, Sdr (serine-aspartate repeat domain) proteins, fibronectin-binding proteins (Fnbp) A and B and clumping factors A and B (Joh *et al*., 1999). CWA proteins are covalently anchored to cell wall by sortase which cleaves sorting signals at the LPXTG motif (Mazmanian *et al.*, 2001), and have been shown to be important in the pathogenesis of infections associated with indwelling medical devices and infective endocarditis (Lowy, 1998). *S. aureus* can invade many different cell types, through a fibronectin bridge which is formed between FnbpA or B and host α5β1 integrin receptors which leads to internalisation (Schwarz-Linek *et al.*, 2003, Peacock *et al.*, 1999, Schwarz-Linek *et al.*, 2004). Small colony variants have been shown to survive within host cells in a semi dormant state (von Eiff *et al.*, 2000).

Secreted virulence determinants including tissue degrading enzymes, cytotoxins, SAgs and exfoliative toxins enable the organism to survive within the host, and contribute to *S. aureus* virulence (Bohach., 2006). A large number of extracellular enzymes such as coagulase, numerous proteases, nuclease and lipase are produced, which can degrade organic macromolecules to provide low molecular weight nutrients for the bacterium, or degrade tissue constituents to facilitate bacterial spread (Arvidson, 2006). Proteolytic activity varies between strains, however most human isolates harbour genes encoding one serine protease (V8), one

metalloprotease (aureolysin) and two cysteine proteases (staphopain A and B) (Arvidson, 2006). In addition, *S. aureus* produces a number of immune evasion factors, including a chemotaxis inhibitory protein (CHIPS), which interferes with the complement system by binding formyl peptide receptor and C5a receptor, and staphylococcus complement inhibitor (SCIN) which binds C4bC2a and C3bBb inhibiting formation of C3b (Lee *et al.*, 2004, de Haas *et al.*, 2004). About 60% of human *S. aureus* strains harbour the *chips* and *scin* genes however there is a very low incidence in ruminant isolates (Sung *et al.*, 2008), consistent with their human-specific activity (van Wamel *et al.*, 2006).

Another group of virulence factors are the secretable expanded repertoire adhesive molecules (SERAMs), which include extracellular adherence protein (Eap), extracellular fibrinogen binding factor (Efb) and von Willebrand factor binding protein (vWbp) (Hauck & Ohlsen, 2006). Eap binds to ICAM-1 on endothelial cells and reduces neutrophil migration (Chavakis *et al.*, 2002). Eap induces proliferation of human peripheral blood mononuclear cells (PBMC), with proinflammatory cytokine release (Scriba *et al.*, 2008). The crystal structure of Eap is homologous to the C-terminal of SAgs (Geisbrecht *et al.*, 2005). However Eap is not a SAg, but non-specifically cross-links MHC class II molecules (Massey *et al.*, 2007), and at high concentration has been shown to inhibit T-cell activation by TSST-1 (Haggar *et al*., 2005).

Another important group of toxins are cytolytic and contribute to host cell damage by pore formation in the membrane, resulting in cell lysis and release of internalised bacteria (Foster, 2005). Cytolytic toxins include α-, β-, γ-, and δ-toxins, which lyse neutrophils allowing *S. aureus* to evade the innate immune response and form abscesses (Foster, 2005). α-toxin is secreted as a monomer and assembles into a heptamer forming a β-barrel pore in the membrane (Montoya & Gouaux, 2003) Most *S. aureus* strains harbour the *hla* gene encoding α-toxin, but expression is variable (Bohach., 2006). Animal infection studies have shown that strains expressing high levels of the toxin gene are more virulent than their isogenic derivatives (Bubeck Wardenburg *et al.*, 2007). Bi-component leukotoxins are another class of toxin which are secreted as two separate subunits and assemble into oligomers in the cell membrane, and include γ-toxin and PVL, that can stimulate

and lyse neutrophils and macrophages (Bohach., 2006). γ-toxin gene is present in about 90% of strains whereas *pvl* was found in only 2% of strains in total, but in 98% of CA-MRSA isolates (Peacock *et al.*, 2002, Moran *et al.*, 2006). Exfoliative toxins (ET), A, B and D are associated with SSS and bullous impetigo. ETs bind desmoglein-1, a glycoprotein in the epidermis leading to disruption of the stratum granulosom (Plano, 2004, Ladhani, 2003).

1.6 Superantigens

The term 'superantigen' (SAg) was proposed in 1989 (White *et al.*, 1989, Choi *et al.*, 1989) to describe 2 previously known toxin families including staphylococcal enterotoxins (SEs), the causative agents of food poisoning (Jordan, 1931), and streptococcal pyrogenic exotoxins (SPEs) responsible for scarlet fever (Dick & Dick, 1924) and streptococcal toxic shock (Stevens *et al.*, 1989). SAgs are proteins which bypass the conventional antigen processing pathway, by binding to MHC class II molecules of antigen-presenting cells and the variable region of the T-cell β chain (Vβ) outside of the antigen binding groove (Figure 1.1) (Peavy *et al.*, 1970, Fraser, 1989, Kappler *et al.*, 1989, Dellabona *et al.*, 1990, Jardetzky *et al.*, 1994) (Choi et al., 1989, Dellabona *et al.*, 1990). In so doing, SAgs can react with all T-cells expressing reactive Vβ regions (up to 20%), instead of the normal responding frequency to an antigen of approximately 0.01% (Figure 1.1), resulting in massive inflammatory cytokine release leading to toxic shock (Drake & Kotzin, 1992). SAgs also have the capacity to cause immune suppression through T-cell anergy or deletion (Kawabe & Ochi, 1990).

1.6.1 Bacterial SAgs

S. aureus is known to produce at least 21 distinct SAgs, including SEA - E, SEG - J, SER – T, SElK- Q, U, V, and toxic shock syndrome toxin, TSST-1 (Table 1.1) (Lina *et al.*, 2004, Ono *et al.*, 2008b, Thomas *et al.*, 2007). *Staphylococcus pseudintermedius* produces a canine variant of SEC and SE-int (Edwards *et al.*, 1997, Futagawa-Saito *et al.*, 2004). 11 SAgs have been identified in *Streptococcus pyogenes* including, SPEA, SPEC, SPEG to M, streptococcal superantigen A (SSA), and streptococcal mitogenic exotoxin Z (SMEZ) (Fraser & Proft, 2008).

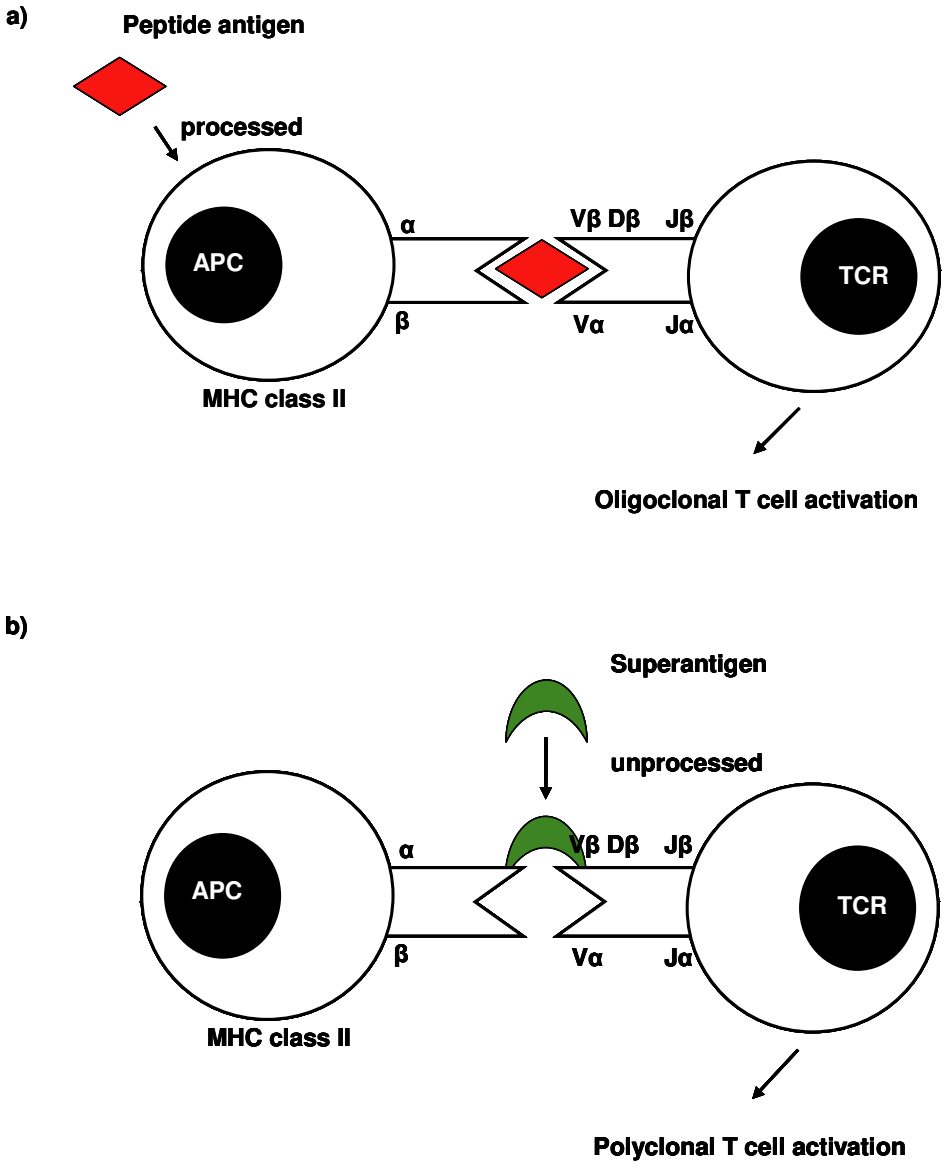

Figure 1.1 Schematic diagram of SAg activity. a) The interaction between conventional antigen presented by the MHC class II presentation pathway and the TCR which leads to the oligoclonal activation of approximately 1:10000 T-cells. **b)** The interaction of SAg with MHC class II molecule and the TCR, which leads to polyclonal activation of approximately 20% of all T-cells.

S. dysgalactiae produces a variant of SPE-G, and *S. dysgalactiae* mitogen (SDM) (Fraser & Proft, 2008). In addition *Streptococcus equi* produces SePE-H, I, Lse, and Mse (Paillot *et al.,* Fraser & Proft, 2008).

Staphylococcal and streptococcal SAgs are short secreted proteins of 21 to 28 kDa, (Thomas., 2007, Petersson *et al.*, 2004), which share similar biochemical and structural properties (Dinges *et al.*, 2000, Thomas *et al*., 2007). With the exception of TSST-1, SAgs are resistant to most proteolytic enzymes and can function in the digestive tract after ingestion (Thomas., 2007). In addition, they are highly resistant to heat, and prolonged autoclaving is required to eliminate function (Schantz *et al.*, 1965). SAgs can be differentiated into 5 distinct subgroups according to their phylogenetic relatedness (Figure 1.2). Surprisingly, some staphylococcal SAgs such as SEH are more phylogenetically similar to streptococcal SAgs, which is likely to be due to HGT between the species. Staphylococcal and streptococcal SAgs have many biological features in common, but one feature which appears to be distinct to staphylococcal SAgs is their ability to transcytose across the epithelium (Hamad *et al.*, 1997). This could explain why TSS but not STSS has been observed in non-invasive disease (Sriskandan *et al.*, 2007).

A further 2 bacterial SAgs, *Mycoplasma arthriditis* mitogen (MAM) and *Yersinia pseudotuberculosis* mitogen (YPM), are phylogenetically and structurally unrelated to this group (Thomas *et al*., 2007, Orwin *et al.*, 2002, Thomas *et al.*, 2009). MAM is responsible for a shock-like disease in rats, mice and rabbits, and has also been shown to be associated with arthritis in rats (Cole *et al.*, 1981, Cole *et al.*, 1971). There is evidence to suggest that a soluble form of the M1 protein of Group A streptococcus (GAS) has superantigenic activity (Cunningham, 2000). M protein is a surface protein which can be released via host- or bacterium-derived protease activity. M1 strains have been epidemiologically associated with invasive streptococcal infections such as STSS (Cunningham, 2000).

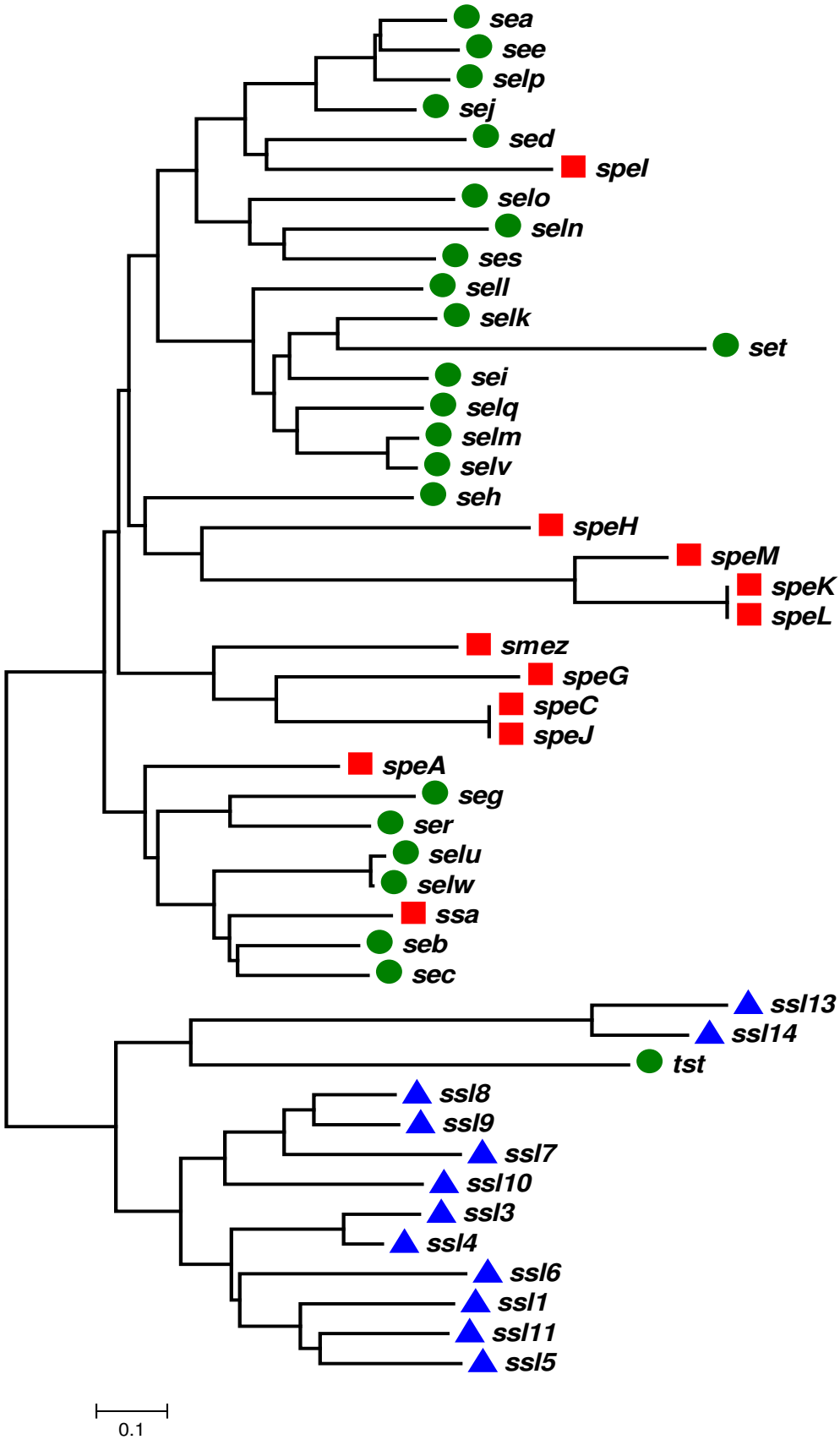
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Figure 1.2: Phylogenetic analysis of SAgs produced by *S. aureus* and *S. pyogenes* and SSLs. Sequences were obtained from the NCBI Genbank database. Phylogenetic tree was constructed using MEGA (Molecular Evolutionary Genetics Analysis) software, from a ClustalW multiple amino acid alignment of the nucleotide sequences. Evolutionary distances were determined by constructing Neighbour Joining trees using the Maximum Composite Likelihood method. At least 500 bootstrap trees were generated to examine the stability of the phylogenetic relationship. Green circles indicate *S. aureus* SAgs, red squares, streptococcal SAgs, and blue triangles, SSLs.

Table 1.1: Properties of characterised staphylococcal SAgs

SAg	Molecular mass (kDa) [a]	Emetic [b]	Human VB specificity [c]	Genomic Location
SEA	27.1	+	1, 5, 6, 7, 9, 15, 16, 18, 21, 22, 24	ΦSa3n
SEB	28.3	+	3, 12, 13, 14, 15, 17, 20	SaPI
SEC	27.5	+	3, 12, 13, 14, 15, 17, 20	SaPI
SED	26.4	+	1, 3, 5, 8, 9, 12, 14	Plasmid (pF5)
SEE	26.4	+	5, 6, 8, 9, 13, 16, 18, 21	Integrated plasmid
SEG	27	+	3, 12, 13, 14, 15	vSAβ
SEH	25.2	+	6, Vα8	transposon
SEI	24.9	+	1, 5, 6, 23	vSAβ
SEJ	28.5	+	8, 21	Plasmid (pF5)
SElK	26	-	1, 5, 6	SaPI/ ΦSa3n
SElL	26	-	1, 5, 7, 16, 22, 23	SaPI
SElM	24.8	NK	8, 9, 18, 21	vSAβ
SElN	26.1	NK	7, 8, 9, 17	vSAβ
SElO	26.7	NK	5, 7	vSAβ
SElP	27	-	5, 8, 16, 18, 21	ΦSa3n
SElQ	28	-	6, 21	SaPI/ ΦSa3n
SER	27	+	3, 12, 14	Plasmid (pF5)
SES	26.2	+	9, 16	Plasmid (pF5)
SET	22.6	+	-	Plasmid (pF5)
SElU	27.1	NK	13, 14	vSAβ
SElU2/W	26.7	NK	NK	vSAβ
SElV	25	NK	6, 18, 21	vSAβ
TSST-1	22	-	2	SaPI

[a] known or predicted.
[b] NK, not known.
[c] data compiled from the references (Seo *et al.,* 2010 Thomas *et al.*, 2009, Ono *et al.*, 2008)

1.6.2 Endogenous SAgs

Superantigenic activity was recognised as similar to minor lymphocyte-stimulating antigens (Mls) (Buxser & Vroegop, 1988). Mls are endogenous SAgs, such as the mouse mammary tumour virus (MMTV) products which were incorporated into the mouse genome, and facilitated the deletion of T-cells as they matured in the thymus, thereby preventing the host from mounting an appropriate T-cell response against gut B cells infected with MMTV (Choi *et al.*, 1991, Marrack *et al.*, 1991, Acha-Orbea & Palmer, 1991, Woodland *et al.*, 1991). MMTV-related retroviral endogenous SAgs in humans have been implicated in type II diabetes (Conrad *et al.*, 1997).

1.7 Staphylococcal superantigen like toxins (SSLs)

SSLs are toxins produced by *S. aureus* which display significant sequence and structural homology with SAgs. However they are not mitogenic for T-cells and do not bind MHC class II (Langley *et al.*, 2005, Williams *et al.*, 2000, Fitzgerald *et al.*, 2003). Instead they have been shown to target the innate immune system in various ways (Fraser & Proft, 2008). *ssl* genes 1 to 11 are located on the highly variable chromosomal region vSaα, with *ssl* 12 to 14 located 0.7 MB downstream of the main cluster (Fraser & Proft, 2008). vSaα corresponds with RD13, one of 18 large chromosomal regions of difference identified by comparative genomic hybridisation (Fitzgerald et al., 2001b). This region was identified in all strains and varied in size from 12 to 17 kb, encoding at least 7 SSLs (Fitzgerald *et al.*, 2003). It has been shown that SSL7 enhances survival at mucosal surfaces due to an ability to block binding to the FcαR1 portion of IgA (Langley *et al.*, 2005). In addition the β-grasp domain of SSL7 binds complement factor C5 and inhibits complement-mediated haemolytic activity (Langley *et al.*, 2005). SSL5 and SSL11 target neutrophils, through a glycan-binding site in the C-terminal domain, which is specific for tri-saccharide sialyl-lactosamine (sLacNac), a ligand that regulates neutrophil adherence to endothelial cells, and is expressed on cell surface glycoproteins such as P-selectin glycoprotein (PSGL-1) (Baker *et al.*, 2007, Chung *et al.*, 2007). Recombinant SSL5 and SSL11 have been shown to inhibit neutrophil adherence and become internalised by neutrophils (Bestebroer *et al.*, 2007, Chung *et al.*,

2007). The sLacNac binding site is also present on SSL2, 3, 4, 5, 6 and 11 (Baker *et al*., 2007).

1.8 SAg activity

The consequences of SAg activity during *S. aureus* infection are not fully understood. SAgs are potent toxins which are responsible for massive polyclonal activation of T-cells, rather than the typical focussed T-cell response to foreign antigens. Rapid expansion of T-cells leads to systemic release of pro-inflammatory cytokines, including interleukin-1 (IL-1), tumour necrosis factor- α (TNF-α), TNF-β, and interferon-γ (IFN-γ) (Langford *et al.*, 1978, White *et al*., 1989). The release of TNF-α and IL-1 is responsible for symptoms associated with TSS such as hypotension, shock and fever (Petersson *et al*., 2004). SAg interference with the immune response to infection can lead to a number of detrimental effects within the host including, pyrogenicity (Clark & Borison, 1963), lethal shock (Crawley *et al.*, 1966) and increased susceptibility of the host to endotoxin (Sugiyama *et al.*, 1964, Dinges *et al.*, 2000). The mechanism of endotoxin and SAg synergy established *in vitro* involves SAg-MHC II interaction followed by IFN-γ release by T-cells which activates TLR4 (Hopkins *et al.*, 2008, Dalpke & Heeg, 2003). In turn TLR4 recognition of LPS leads to LPS-enhanced MHC class II expression on APC (Jephthah-Ochola *et al.*, 1988). SAgs have been shown to suppress the immune system through T-cell anergy or deletion (MacDonald *et al.*, 1991, Wang *et al.*, 1998, Kawabe & Ochi, 1990), modulation of apoptosis (Boshell *et al.*, 1996, Alderson *et al.*, 1995), and the induction of regulatory T cells (T_{regs}) (Sundstedt *et al.*, 1997, Zheng *et al.*, 2002, Seo *et al.*, 2009). Additionally they have been proposed to play a role in autoimmunity, due to the stimulation of T-cells reactive to self-antigens (Thomas *et al*., 2007).

Staphylococcal SAgs which have the capacity to induce an emetic response are classified as staphylococcal enterotoxins (SEs), and those which lack emetic properties or have not been tested are designated as staphylococcal enterotoxin-like toxins (SEls) (Lina *et al*., 2004, Ono *et al*., 2008, Thomas *et al*., 2007). Despite intensive research efforts, the mechanism by which SEs induce emesis is still unclear. A disulphide loop located in the N-terminus was thought to be associated

with emetic activity, but recent studies using site directed mutagenesis have observed only a reduction in emesis rather than elimination (Wang *et al.*, 2009) (Hovde *et al.*, 1994). More recently it has been proposed that SEs and not SE*l*s are able to penetrate the gut lining, activating a local and systemic immune response with the release of inflammatory mediators including histamine which could be responsible for GI tract damage and emesis (Shupp *et al.*, 2002, Thomas *et al.*, 2007).

1.9 SAg structure

Crystallography has revealed the structure of a number of SAgs including staphylococcal SAgs, SEA, B, C2, C3, D, G, H, I, K, and TSST-1, streptococcal SAgs SPEA, C, H, I, J, SMEZ-2 and SDM, MAM and YPM (Brouillard *et al.*, 2007, Papageorgiou *et al.*, 1999, Roussel *et al.*, 1997, Arcus *et al.*, 2000, Baker *et al.*, 2004, Saarinen *et al.*, 2007, Swaminathan *et al.*, 1992, Schad *et al.*, 1995, Papageorgiou *et al.*, 1995, Fields *et al.*, 1996, Sundstrom *et al.*, 1996, Fernandez *et al.*, 2006b, Marisa M. Fernández, 2007, Hakansson *et al.*, 2000, Gunther *et al.*, 2007, Acharya *et al.*, 1994). Staphylococcal and streptococcal SAgs share a compact 2-domain globular protein structure including domain A which contains a long central α-helix, and a C-terminal β-grasp motif. Domain B is smaller and contains a mixed β-barrel or oligonucleotide-oligosaccharide binding-fold (OB) (Murzin, 1993) (Figure 1.3). The OB fold is a common domain in heat labile enterotoxins such as verotoxin, pertussis and cholera toxins, members of the AB_5 toxin family (Mitchell *et al.*, 2000). Close packing of the domains and extension of the N-terminus over the top of the C-domain underlies the impressive stability of SAg proteins. The disulfide bond which has been implicated in emetic activity is located at the top of domain B (Krakauer, 2005). YPM and MAM are structurally unrelated to staphylococcal and streptococcal SAgs. YPM is a single domain structure with a jelly roll fold of 2 β-sheets, and MAM consists of 10 α-helices which form an L-shaped protein fold (Donadini *et al.*, 2004, Zhao *et al.*, 2004). The MAM protein binds to the top of MHC class II α-1-helix and β-1 helix, via 2 α-helical bundles (Zhao *et al.*, 2004). Crystal structures of SSL5, SSL7, and SSL11 have also been solved and each display a 2-domain structure analogous to SAgs,

and in particular TSST-1 (Al-Shangiti *et al.*, 2004, Arcus *et al.*, 2002, Chung *et al.*, 2007). However there are notable differences in regions involved in SAg activity such as MHC class II and TCR binding (Fraser & Proft, 2008). The SSLs are characterised by an elongated β6 to β7 loop in the C-terminal domain, through which they crystallise as a homodimer. However the functional significance of this is currently unknown (Fraser & Proft, 2008).

1.10 SAg interactions with the MHC and TCR

SAgs co-ligate MHC class II molecules on the surface of antigen-presenting cells (APC) and TCR molecules, but simultaneous binding is considered unlikely (Sriskandan *et al.,* 2007). The SAg must first bind to the MHC class II, in order to utilise the adhesion and accessory molecules required to bind to the TCR. SAgs bind to common conserved elements of MHC class II molecules, but preferential binding to certain MHC HLA (human leukocyte antigen) types is exhibited. SEC preferentially binds to HLA-DQ, a cell surface protein found on antigen presenting cells, whereas SEA, SEB, SED, SEE, SEH and TSST-1 preferentially bind HLA-DR (Herrmann *et al.*, 1989). The strength of binding between SAg and HLA class II is variable depending on the HLA allele, which may impact on the level of T-cell stimulation (Llewelyn *et al.*, 2004). HLA haplotypes are also implicated in susceptibility to severe shock caused by GAS (Kotb *et al.*, 2002). Information on the HLA-binding affinity of many recently identified SAgs has not yet been determined. SAgs have a higher affinity to MHC class II molecules than towards TCR (Pless *et al.*, 2005).

When the surface concentration of bound SAg on the APC is sufficient to cross-link multiple TCR molecules, a TCR signalling cascade is activated leading to rapid cytokine production (Pless *et al*., 2005). The affinity and binding rates of SAg/TCR/MHC complexes are comparable to those measured for TCR/peptide/MHC complexes (Sundberg *et al.*, 2007, Petersson *et al*., 2004). SAgs can be divided into 3 distinct groups based on their MHC class II binding mechanism; binding to the MHC class II α-chain (e.g SEB and TSST-1), zinc-mediated binding to MHC class II β-chain (e.g SEH and SEI) or the ability to bind to both α- and β-MHC class II chains (e.g SEA) (Tiedemann & Fraser, 1996,

Jardetzky et al., 1994, Fernandez *et al.*, 2006a, Petersson *et al.*, 2001, Petersson et al., 2004, Kim *et al.*, 1994, Tiedemann *et al.*, 1995). SEB and TSST-1 bind to MHC class II α-chain via their OB fold (Jardetzky et al., 1994, Kim et al., 1994). SPEC binds to the MHC class II β-chain via a C terminal Zinc binding site (Li *et al.*, 2001). It has been shown that a strong MHC class II/SAg interaction is required for the extreme potency of SAgs, as murine PBMC require a 5-fold higher dose of SAg than human PBMC for mitogenicity (Fraser & Proft, 2008). Mice transgenic for human MHC class II are significantly more sensitive in their T-cell proliferation responses, confirming that SAgs have a higher affinity for human than murine MHC class II (Nooh *et al.*, 2007, Sriskandan *et al.*, 2001). SAgs primarily interact with the TCR by binding to the variable region of the T-cell β chain (Vβ) region, with the exception of SEH which recognises the variable region of the TCR α-chain (Pumphrey *et al.*, 2007). The TCRVα region is thought to indirectly influence the specificity for SAg by interaction with the β-chain of MHC (Petersson *et al.*, 2004).

Crystallography studies have revealed that SAgs bind to the complementary determining region (CDR) loop 2 domain of the TCR (Sundberg *et al.*, 2007). The SAg TCR binding site is a cleft between the N-terminal and C-terminal domains (Figure 1.3). The interface between domains is the only highly conserved sequence amongst all staphylococcal and streptococcal SAgs and SSLs characterised to date (Fraser & Proft, 2008). SEB, SEC, SPEA binding to the TCR is CDR2 and hyper-variable region (HVR) 4 restricted and is independent of specific Vβ amino acids (Sundberg *et al.*, 2002b). Specificity is achieved through binding Vβ hypervariable loops CDR1, CDR2, CDR3 and HVR4 (Li *et al.*, 1998). It has been suggested that SAgs can be divided into either promiscuous TCR binders or highly specific T-cell activators with a narrow Vβ profile (Sundberg *et al.*, 2002a). However further experiments will be required to investigate this hypothesis properly. The SAg/TCR binding mechanism is generally thought to be similar for distinct SAgs (Petersson *et al.*, 2004).

1.11 The T-cell receptor (TCR)

The TCR is a heterodimer consisting of α- and β- or γ- and δ-chains. The αβ TCR repertoire is associated with very high levels of diversity to allow the recognition of

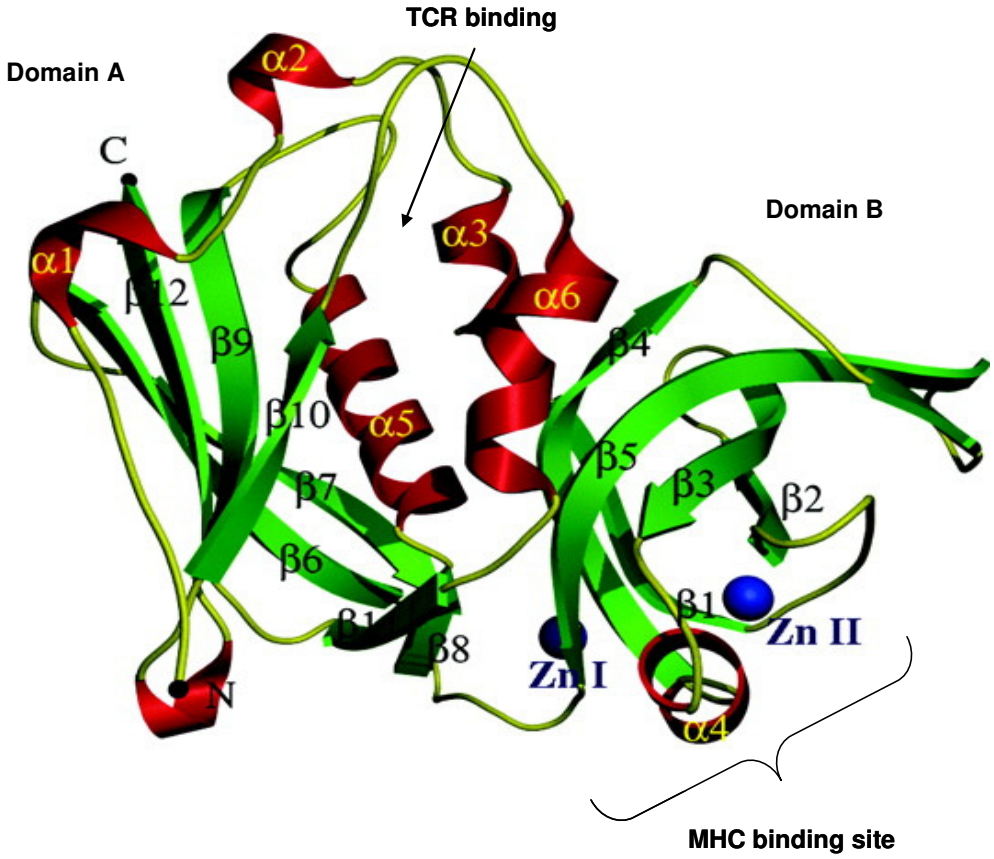


Figure 1.3: Ribbon diagram of SEC2 structure. α-helices are shown in red and β-helices are shown in green. Zinc ions are shown in blue (Papageorgiou *et al.*, 2004).

the large number of MHC ligands presented to T-cells (Nikolich-Zugich *et al.*, 2004). Diversity can be generated by somatic recombination, whereby individual variable (V), diversity (D), and joining (J) segments are combined to form the single exons encoding the variable regions of α- and β-chains. Combinatorial joining of V-J and V-D-J region gene segments generates diversity within TCR β chain CDR loop region 3 (Davis & Bjorkman, 1988, Rowen *et al.*, 1996). Imprecise joining during VDJ recombination and insertion of palindromic nucleotides within the VD, DJ, and VJ region leads to further diversification (Behlke *et al.*, 1985, Marrack & Kappler, 1997). CDR1 and CDR2 sequences within the TCR interact with the α-helix of the MHC class II molecule (Garcia *et al.*, 1996), and the CDR3 region interacts with the antigenic peptide associated with MHC leading to clonal T-cell proliferation (Garcia *et al*., 1996).

T-cell receptor β variable (TRBV) genes encode the variable region of the β chain, and the full repertoire of human and murine TRBV genes has been established (Rowen *et al*., 1996). In cattle, TRBV genes are designated as orthologues of their most similar human genes, on the basis of over 75% sequence homology to each other and human TRBV genes and are assigned to subgroups accordingly. Vβ sequence homology between orthologous subfamilies from different species is higher than between different subfamilies in the same species which indicates that TRBV gene subfamilies were established before mammalian radiation 60 to 80 million years ago (Clark *et al.*, 1995). The recent bovine genome sequencing project added to the TRBV information obtained from cDNA analyses and revealed that the bovine TRBV locus is around 730 kb in size, containing 134 genes of which 79 are predicted to be functional (Houston & Morrison, 1999, Elsik *et al.*, 2009). These genes can be assigned to 24 subgroups of which 18 contain functional genes (Connelley *et al.*, 2009). Organisation of the bovine TRBV locus is broadly similar in humans and mice. However the bovine repertoire is much larger, with human and murine TRBV loci encoding 65 genes belonging to 30 subgroups, and 35 genes belonging to 31 subgroups, respectively (Rowen *et al*., 1996, Folch & Lefranc, 2000, Bosc & Lefranc, 2000). Therefore it appears that the cattle TRBV gene repertoire is distinct from other species and indicates that there has been extensive gene duplication in the TRBV locus during evolution (Houston *et al.*,

2005). It is thought that expansion of genomic TRBV evolved by extensive gene duplication involving tandem duplication of DNA blocks containing multiple genes (Su & Nei, 2001). In particular, there has been massive expansion of bovVβ subfamilies 1, 10 and 13 containing 35, 16 and 40 genes respectively, comprising 68% of all bovine TRBV genes (Connelley *et al*., 2009).

1.12 SAg stimulation of bovine T lymphocytes

Of the SAgs produced by *S. aureus,* SEA, SEB, SEC and TSST-1 have been shown to be mitogenic for bovine T lymphocytes (Deringer *et al.*, 1997, Seo *et al.*, 2007, Fitzgerald *et al.*, 2001a, Yokomizo *et al.*, 1995), but their contribution to disease pathogenesis has not been investigated in detail. Interestingly, TSS has not been described in cattle, despite the high incidence of infection with SAg-producing *S. aureus* strains, which suggests that the bovine immune system may be distinct in its response to SAg stimulation (Park Y.H, 2006). Bovine T-cell proliferation on exposure to SAgs has been shown to be Vβ-dependent. Deringer *et al,* examined the response of 5 bovine Vβ subfamilies in response to stimulation with SEC_{bov} and found Vβ 28 T-cells were selectively expanded (Deringer et al., 1997). The Vβ specific expansion of bovine lymphocytes in response to stimulation with culture supernatants of a bovine strain of *S. aureus* and *tst*- and *secbov*-deficient isogenic mutants, was also investigated by Fitzgerald *et al*. TSST-1 was shown to activate bovVβ 2, consistent with the effect of the toxin on human T-cells (Fitzgerald *et al*., 2001a). Vβ 1 and Vβ 7 levels were elevated in the absence of *tst* and *secbov*, which suggested expression of other SAgs by strain RF122 (Fitzgerald *et al*., 2001a).

1.13 Distribution of staphylococcal SAgs

All SAgs identified to date are encoded MGEs such as plasmids, phages, transposons and staphylococcal pathogenicity islands (SaPIs), or the highly variable genomic region vSaβ (Table 1.1) (Fitzgerald et al., 2001a, Jarraud *et al.*, 2001, Johns & Khan, 1988, Ono *et al*., 2008, Ben Zakour *et al.*, 2008). For example, *sea* and *selp* are encoded by phage ΦSa3n, and *sed, sej, ser, ses,* and *set* are carried by a plasmid (Ono *et al*., 2008, Zhang *et al.*, 1998). The enterotoxin gene cluster (*egc*) is located at a highly variable region of the chromosome vSaβ and consists of five

genes, *seg*, *sei*, *selo*, *selm*, *seln* and either two pseudogenes φ*ent1* and φ*ent2* or *selu* (Letertre *et al.*, 2003, Jarraud *et al.*, 2001). vSaβ is found in all strains however the presence of the *egc* is strain-dependent. The *S. aureus* pathogenicity islands (SaPIs) encode a number of genes associated with virulence, including, *seb*, *sec*, *selk*, *sel selq* and *tst* (Novick *et al.,* 2010*)*. SaPIs range in size from 14 kb to 17 kb, contain a site-specific integrase, are capable of autonomous replication, and can be transferred horizontally between strains (Novick *et al.,* 2010*)*. Transfer relies on induction by bacteriophages to initiate an excision-replication-packaging mechanism resulting in incorporation of the genomic island into infective phage-like particles (Ubeda *et al.*, 2007). Accordingly, the distribution of SAg genes among *S. aureus* strains is highly variable. For example, Omoe *et al*, showed that 80% of human nasal isolates contain at least one SAg gene, including 50% which contain the *egc* locus, and Smyth *et al* demonstrated that 57% of animal-associated strains examined contained at least one SAg gene with the *egc* found in 30% of isolates (Smyth *et al.*, 2005, Omoe *et al.*, 2005a). These data imply that no single SAg is encoded by more than 50% of strains and that about a fifth of all strains may not have superantigenic capacity at all. Staphylococcal SAgs have also been described in coagulase negative staphylococci (CNS) including; *S. chromogenes*, *S. xylosis*, *S. hyicus*, *S. simulans*, *S. epidermidis*, and *S. haemolyticus*. Park *et al* demonstrated that 31% of CNS isolates tested harboured at least one SAg gene (Park *et al.,* 2010*)*. In addition a high proportion of *S. pseudintermedius* strains harbour the SAg gene *seint* and a small number of *S. pseudintermedius* strains produce a canine variant of SEC (Edwards *et al*., 1997, Futagawa-Saito *et al*., 2004).

There are a number of studies which suggest that specific combinations of SAg genes are linked to different clinical outcomes. For example, *sea* and *sej* have been associated with invasive disease, *sea*, *seb*, and *tst* are more frequently observed in sepsis isolates and the *egc* genes are thought to predominate in commensal strains (Peacock *et al*., 2002, Ferry *et al.*, 2005). However it has been reported that specific gene combinations often occur as a result of the clonal origin of the strains (Holtfreter *et al.*, 2007). Holtfreter *et al* investigated the distribution of SAg encoding MGEs in a wide range of human MSSA and MRSA isolates, and observed SAg gene complement was tightly linked to specific clonal complexes. The *egc* was

present in all CC5, CC22 and CC45 isolates, and *tst* was strongly linked to the CC30 lineage. However, broader distribution was observed for the *sea* carrying phage, ΦSa3n implying wide horizontal transfer (Holtfreter *et al*., 2007).

1.14 Staphylococcal gene regulation

The survival of *S. aureus* in the environment and within various niches in a variety of animal hosts relies on a number of adaptive gene regulatory systems. In particular, the accessory genes of *S. aureus* are controlled by an intricate regulatory network comprised of two component signal transduction systems (TCS) and transcription factors including alternative sigma factors and the Sar (Staphylococcal accessory regulator) protein family of homologous DNA binding proteins. This network allows the bacterium to produce the appropriate accessory or virulence factors it needs in response to environmental or population density stimuli. Analysis of the *S. aureus* genome revealed at least 16 TCSs in *S. aureus* (Cheung *et al.*, 2004, Novick, 2003). The accessory gene regulator (*agr*) is a well characterised staphylococcal TCS consisting of 4 genes which encode components of a quorum-sensing system activated at high cell density (Novick, 2003). AgrA and AgrC form the TCS, and AgrB and AgrD produce an autoinducing peptide (AIP) for activation (Novick, 2003). AIP is post-translationally modified from AgrD which is a propeptide, and binds to the *agr* signal receptor AgrC (Ji *et al.*, 1995, Lina *et al.*, 1998). The *agr* locus contains two separate transcripts, RNAII and RNAIII driven by promoters P2 and P3 repectively. RNAII encodes *agrDBCA*, the TCS and its autoinducing ligand, RNAIII transcript encodes a regulatory molecule (RNAIII) and *hld* (δ-toxin). When the response regulator, AgrA is activated by phosphorylation, P2 and P3 promoters are upregulated. Intracellular accumulation of RNAIII leads to increased transcription of exotoxins (Novick, 2003). Four major *agr* groups have been described based on sequence divergence within the locus (Wright *et al.*, 2005, Novick, 2003). The virulence of *agr* mutants is attenuated in a number of animal models, therefore *agr* has been well established as a global regulator of virulence (Novick, 2003, Abdelnour *et al.*, 1993, Gillaspy *et al.*, 1995)

The *sarA* locus is controlled by 3 upstream promoters, and can produce 3 distinct transcripts encoding the SarA protein (Cheung *et al.*, 2004). SarA is a

transcriptional factor which upregulates expression of RNAIII through interaction with, or independent of the *agr* (Chien & Cheung, 1998, Dunman *et al.*, 2001). The rot (repressor of toxin) locus is a member of the Sar family which interacts with the *agr* (McNamara *et al.*, 2000). Inactivation of rot restored α-toxin and protease expression in an *agr* null mutant (McNamara *et al*., 2000). Rot is a global regulator of virulence factors including protease, alpha haemolysin, protein A and clumping factor (Said-Salim *et al.*, 2003). Overall, this complex gene regulatory network allows differential expression of proteins in different phases of bacterial growth. During exponential phase, expression of many adhesive CWA proteins occurs (Cheung *et al*., 2004, Xiong *et al.*, 2004). During post-exponential phase extracellular toxins such as SAgs, α-toxin and proteolytic enzymes are upregulated, which are likely to facilitate nutrient acquisition and dissemination (Cheung *et al*., 2004). Inactivation of either *sarA* or *agr* loci leads to reduced virulence in animal infection models (Cheung *et al*., 2004, Xiong *et al*., 2004).

1.15 Expression of SAg genes

Gaskill *et al* demonstrated that *seb* was under the control of the *agr*, as SEB production was lower in an *agr* null mutant (Gaskill & Khan, 1988). *In vitro* experiments have shown that in addition to *seb, agr* upregulates the expression of genes encoding SEC, SED and TSST-1 in stationary phase (Tseng *et al.*, 2004, Regassa *et al.*, 1991, Novick, 2003). However this does not appear to be the case for all SAg genes, as SEA is known to be constitutively expressed and SEG and SEI are produced maximally in exponential phase (Lina., 2004, Munson *et al.*, 1998b, Tremaine *et al.*, 1993). TSST-1 and SEB have themselves been reported to function as transcription factors, acting as global repressors of exoprotein genes at the level of transcription (Vojtov *et al.*, 2002b). Expression of TSST-1 has also been confirmed *in vivo*, and TSST-1 toxin levels of up to 5 ng/ml have been detected in sera from TSS patients (Miwa *et al.*, 1994). In addition, expansion of Vβ 2 lymphocytes by TSST-1 was observed in TSS patients (Takahashi *et al.*, 2000).

Some SAgs encoded by *S. pyogenes* such as SPEA, are produced at greater levels in BALB/c mice compared to liquid culture *in vitro*, indicating that optimal expression relies on unknown host factors (Kazmi *et al.*, 2001). In addition, SPEC

expression was significantly increased in co-culture with human pharyngeal cells (Broudy *et al.*, 2001). Another important study which monitored cynomolgus macaques throughout the course of *S. pyogenes* infection, revealed maximal *spej* and *smez* transcription during colonisation, and *spej* expression during early infection stages (Virtaneva *et al.*, 2005). The closely related SSLs are also produced at low levels *in vitro* (Fraser & Proft, 2008), but are dramatically upregulated under stress conditions, in a hemin-fed strain of *S. aureus* which is deficient in *hrtAB,* required for iron efflux (Torres *et al.*, 2007).

1.16 Toxic shock syndrome (TSS)

TSS is an acute disorder which was first identified in children in 1978, and is characterised by high fever, vomiting, diarrhoea, and an erythrematous rash, which progresses to more severe disease with hypotension, shock and renal failure (Todd *et al.*, 1978). In 1980, 400 cases were reported to the Centers for Disease Control (CDC) (Bergdoll *et al.*, 1981). Almost all of the cases during the initial US outbreak were associated with menstruating females infected with a phage group I strain of *S. aureus* (Bergdoll *et al*., 1981). 88% of all subsequent menstrual cases examined from 5 countries were attributed to a single clone of *S. aureus* (Musser *et al.*, 1990). Bergdoll *et al* showed that 94% of menstrual TSS isolates produced high levels of TSST-1 compared with only 4% of non-TSS strains (Bergdoll *et al.*, 1981). TSST-1 is thought to be the only SAg able to penetrate the vaginal mucosa, by binding directly to vaginal epithelial cells and activating innate chemokines to allow penetration into the bloodstream (Peterson *et al.*, 2005). Therefore TSST-1 is generally considered to be the only SAg responsible for menstrual toxic shock (Thomas *et al*., 2007).

A further link to the use of a particular brand of highly absorbent tampons (Rely) amongst TSS patients was discovered (Kehrberg *et al.*, 1981, Schlech *et al.*, 1982). The removal of that brand of tampons from the market and changes in tampon manufacture led to a significant reduction in the incidence of menstrual TSS (Schuchat & Broome, 1991). Since then, non-menstrual TSS which affects a heterogenous patient group has been more commonly observed, and in addition to TSST-1, is associated with strains producing SEA, SEB, and SEC (Ferry *et al.*,

2005, Dauwalder *et al.*, 2006). While menstrual TSS rates have fallen, non-menstrual TSS rates have remained stable (Schlievert *et al.*, 2004, Hajjeh *et al.*, 1999). Recurring TSS is thought to be associated with a failure to generate neutralizing antibodies due to immuno-depression caused by SAgs (Andrews *et al.*, 2001).

1.17 Other SAg-associated diseases

Leydon *et al* first proposed that *S. aureus* may be involved in atopic dermatitis (AD), as it was observed that the skin of almost all patients were colonised with *S. aureus* compared with only 5% of healthy volunteers (Leyden *et al.*, 1974). A number of studies subsequently reported that AD isolates produced SAgs and a possible role in the progression of AD disease was speculated (Hoeger *et al.*, 1992, Michie & Davis, 1996, Leung & Bieber, 2003). Strickland *et al* later demonstrated that SAgs upregulate the expression of the skin homing receptor, cutaneous lymphocyte associated antigen (CLA), and AD patients showed significant SAg-specific Vβ skewing within the CLA^+ T-cell subsets (Strickland *et al.*, 1999).

A similar association with SAg activity was proposed for allergic rhinitis, as nasal carriage levels in patients (44%) was much higher than in healthy subjects (20%) (Shiomori *et al.*, 2000). Rossi *et al* determined the prevalence of serum-specific IgE towards SEA, SEB, SEC, SED and TSST-1 in patients suffering from rhinitis, and observed an increase in serum eosinophil cationic protein (ECP), which is a marker for the severity of allergic disease, in patients with anti-SAg IgE compared to IgE negative patients (Rossi & Monasterolo, 2004). SAgs have been shown to stimulate immunoglobulin class switching to SAg specific IgE or "superallergens" in mucosal B cells. Superallergens then promote chronic inflammation and persistent sensitisation of mast cells and dendritic cells to conventional allergens, in mucosal tissues of atopic dermatitis, rhinitis, and asthma patients and in nasal polyposis (Bachert *et al.*, 2001, Barnes, 2009).

Another disease which has been linked to SAg activity is Kawasaki disease (KD). KD is an acute disease in children with symptoms similar to TSS (Falcini, 2006), but the etiology of this disease is unknown. Leung *et al* observed Vβ 2-specific T-cell expansion associated with TSST-1 production in a number of

patients (Leung *et al.*, 1993, Leung *et al.*, 1995). Matsabura *et al* described an increase in SAg-specific serum IgM antibodies in patients in the weeks following diagnosis with KD (Matsubara *et al.*, 2006). However further investigations are required to confirm the role of SAgs in KD.

1.18 Therapeutics

The growing problem of antibiotic resistance amongst *S .aureus* isolates has led to increased need to develop alternative therapeutic agents. Currently, there is no vaccine available for *S. aureus*, but a number of clinical trials are ongoing (Garcia-Lara & Foster, 2009). Potential targets include CP5 and CP8 capsular polysaccharides, α-toxin, CWA proteins and SAgs. SAg vaccines which are in pre-clinical development include a toxoid mutant of TSST-1 which has shown protection in rabbits and an SEB toxoid which is protective in monkeys (Garcia-Lara & Foster, 2009). Another therapy in development are peptide agonists, which target regions of SAg which interact with the host to inhibit activity (Arad *et al.*, 2000). Yang *et al* have described a broad spectrum antagonist which consists of multiple Vβ domains expessed in tandem as a single-chain protein which can neutralize SEB and TSST-1 (Yang *et al.*, 2008).

1.19 Hypothesis

SAgs are produced by a large number of *S. aureus* strains and contribute to pathogenesis through modulation of the host immune response. However the exact role SAgs play in the pathogenesis of disease is unclear. Studies have revealed that SAg genes are commonly found in bovine mastitis isolates (Smyth *et al*., 2005). SEC and TSST-1 have been shown to activate bovVβ specific T-cell activation (Fitzgerald *et al*., 2001a), but the effect of other staphylococcal SAgs on bovine immune response has not been investigated. We propose that a number of bovine-specific SAg genes encoded by the bovine *S. aureus* strain RF122 will have a role in activation of the bovine immune response. Chronic, persisting, subclinical infection is the most common form of disease and we believe persistence may be due in part to SAg activity.

1.20 Project aims

The aim of this project was to elucidate the role of staphylococcal SAgs in the pathogenesis of *S. aureus* by:

- Employing a genome scale approach to identify the full complement of SAg genes encoded by bovine strain RF122, and investigate levels of gene expression

- Investigating bovine T-cell activation in response to staphylococcal SAgs

- Examining the role of SAgs in pathogenesis during experimental intra-mammary infection

Chapter 2
General Materials and Methods

2.1 Bacterial culture conditions

S. aureus strains were grown in tryptone soya broth (TSB) or brain heart infusion broth (BHI) (Oxoid, UK) shaken at 200 rpm, or on tryptone soya agar (TSA) (Oxoid, UK) at 37 °C for 16 h unless otherwise stated. *E. coli* strains were grown in Luria-Bertani Broth (LB) (Melford Laboratories, UK) shaken at 200rpm, or on LB-agar (Melford Laboratories, UK) at 37 °C for 16 h unless otherwise stated. Media were supplemented where appropriate with antibiotics as stated in each chapter. Strains were stored in appropriate liquid culture medium containing 40% (v/v) glycerol (Sigma-Aldrich, UK) in cryovials (Nunc, Thermo Scientific, UK) at -80 °C.

2.2 Genomic DNA extraction

Genomic DNA (gDNA) was extracted from 1ml of an overnight culture of *S. aureus* strains using the PurElute™ Bacterial Genomic Kit (Edge Biosystems, MD, USA) as described in the manufacturer's protocol, except for the addition of 100 µg/ml lysostaphin to Spheroplast lysis buffer and incubation at 37 °C for 20 min (AMBI products LLC, NY, USA). gDNA pellets were resuspended in 100 µl 1 x Tris-EDTA (TE) buffer (10mM Tris, 1mM EDTA, adjusted to pH 8) (Sigma-Aldrich, UK).

2.3 DNA isolation

DNA plasmids were isolated from 5 ml overnight cultures of *E. coli* using the QIAprep Spin Miniprep kit (QIAgen) as described in the manufacturers instructions, and from *S. aureus* with the addition of lysostaphin (AMBI products LLC) to Buffer P1 at 100 µg/ml and incubation at 37 °C for 1 h. DNA fragments were purified using QIAquick gel extraction or PCR purification kits from QIAgen as outlined in the supplied protocol.

2.4 Restriction digestion

gDNA and plasmid DNA were digested with appropriate restriction enzymes and buffers as outlined in the manufacturer's instructions (New England Biolabs, Herts, UK). Digestion of 2 µg gDNA was carried out in a 50 µl reaction containing 1 x

reaction buffer, 2 U restriction endonuclease and dH_20 for 16 h. 50 ng plasmid DNA was digested in a 20 µl reaction containing 1 x reaction buffer, 1 U restriction endonuclease and dH_20 for 2 h at 37 °C unless otherwise stated.

2.5 Polymerase Chain Reaction (PCR)

Oligonucleotides were designed using Primer 3 v. 0.4.0 (Rozen, 2000), and synthesised by Sigma-Genosys, UK or Invitrogen, UK. PCR reactions were carried out in a BioRad thermal cycler and contained 100 nM forward and reverse primers as stated in each chapter, 0.25 mM dNTP (Promega, Hampshire, UK), 10 x reaction buffer, and either 1 U of Taq DNA polymerase (Promega, Hampshire, UK), 1 unit of PfuUltra II Fusion DNA Polymerase (Agilent, UK), or 1 U Vent polymerase (New England Biolabs, Herts, UK), 10 ng of template DNA and ddH_2O to a final volume of 50 µl. Colony PCR was carried out by boiling single colonies in 10 µl ddH_2O. Unless otherwise stated, the thermal programme was; 1 cycle at 95 °C for 3 min, 30 cycles at 95 °C for 1 min, 50 °C for 1 min, 72 °C for 30 s per kb (Taq), 15 s per kb (Pfu), or 1 min per kb (Vent), followed by a final extension at 72°C for 10 min

2.6 Agarose gel electrophoresis

Agarose gel electrophoresis of DNA fragments was carried out in 1% (w/v) agarose gels (Melford Laboratories, UK), with 1 x Tris borate EDTA (TBE) buffer (Sigma-Aldrich Co., Dorset, UK) containing either 0.5 µg/ml ethidium bromide (Sigma-Aldrich Co., Dorset, UK) 1 x SYBR Safe DNA gel strain (Invitrogen, UK) or 1 x Gel Red (Biotium Limited, Cambridge Bioscience, UK). 1 x Bluejuice (Invitrogen, UK) or Promega loading dye (Promega, UK) was added to DNA samples before being loaded onto to the gel alongside a molecular weight marker, 100 bp or 1 kb DNA ladder (Promega, UK). Electrophoresis was carried out at 100 V for 60 to 90 min. Gels were visualised using a UV transilluminator (MultiImage™ Light Cabinet, Alpha Innotech Corporation).

2.7 DNA quantification

DNA concentration (ng/µl) was determined using a nanodrop ND-1000 spectrophotometer (Thermo Scientific, USA) by measuring the absorbance of the sample at a wavelength of 260 nm.

2.8 DNA Sequencing

Sequencing reactions were carried out by Genepool Sequencing Service (King's Buildings, University of Edinburgh, UK). Reactions contained 5 µl (approximately 50 ng) purified DNA with 100 nM sequencing primer and the BigDye™ Terminator v3.0 Ready Reaction Cycle sequencing kit as described in the manufacturer's instrucions (Applied Biosystems, UK) and loaded onto an ABI3730 capillary DNA analyzer. DNA sequences were assembled using ContigExpress (Vector NTI Advance 10, Invitrogen, UK) or DNAbaser v.2.80.0 (www.dnabaser.com).

2.9 Phylogenetic analysis

Sequences were aligned by ClustalW using MEGA 4.0.1 software (Tamura *et al.*, 2007). Evolutionary distances were determined by constructing Neighbour Joining trees using the Maximum Composite Likelihood method. At least 500 bootstrap trees were generated to examine the stability of the phylogenetic relationship.

2.10 Cloning of SAg genes

Inserts were amplified by PCR using primers and enzymes as stated in each chapter. PCR products were then purified by either gel extraction or PCR purification, and cloned into the Strataclone pSC-B plasmid as described in the manufacturer's instructions (Stratagene). pSC-B plasmids containing inserts were then restriction digested with appropriate restriction enzymes, purified and ligated into digested plasmid, with T4 DNA ligase (NEB) for 16 h at 4 °C. The ligations were dialysed on 0.025 µm filter discs (Millipore) and transformed into *E. coli* strain DH5α. The constructs were subsequently isolated from *E. coli* and transformed by electroporation into *S. aureus* or *E. coli* expression strain, BL21.

2.11 Preparation of electro-competent cells and electro-transformation

E. coli electro-competent cells were prepared by inoculating 250 µl of an overnight culture into 25 ml of LB shaken at 200 rpm, 37 °C until an OD_{600} of between 0.4 and 0.6 was reached. Cells were washed once in an equal volume of ddH_2O, centrifuged at 4000 x g, 4 °C, washed twice in a 1/2 volume of 10% (v/v) glycerol, and once in 1/4 volume of 10% (v/v) glycerol. Cells were pelleted and resuspended in 10% (v/v) glycerol at a final volume of 400 µl. *S. aureus* cells were made competent by inoculating 1 ml of overnight culture into 25 ml of TSB, followed by shaking at 200 rpm, 37 °C until mid-exponential phase (OD_{600} value of between 0.8 and 1.0). Cells were washed three times in an equal volume of distilled water, washed once in a 1/5 volume of 10% (v/v) glycerol, resuspended in 1/10 volume of 10% (v/v) glycerol and incubated at 20 °C for 15 min. Cells were pelleted and resuspended in a final volume of 800 µl 10% (v/v) glycerol. Electro-transformation was carried out with 1 µg plasmid or ligated DNA added to 70 µl competent cells in a 0.1 cm gap cuvette (Sigma-Aldrich, UK), and electroporated with the following settings; 100 Ω, 25 µF and 2.5 kV. 300 µl TSB or LB was immediately added and cells were shaken at 37 °C for 1 h unless otherwise stated. Cells were plated onto TSA or LB-agar supplemented where appropriate with antibiotics, and incubated for 18 h at 37 °C.

2.12 Sodium Dodecyl Sulphate – Polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were resolved by SDS-PAGE. 10% (w/v) polyacrylamide resolving protein gels were prepared using a mini-protean gel casting apparatus (Biorad) with 4.05 ml of distilled water, 2.5 ml of 1.5 M TrisHCl, pH 8.8 (ForMedium, UK), 100 µl 10% (w/v) Sodium-dodecylsulphate (SDS) (Melford, UK), 3.3 ml 40% (w/v) Acrylamide/Bis-acrylamide (Sigma-Aldrich, UK), 50 µl 10% (w/v) Ammonium persulphate (APS) (Sigma-Aldrich, UK) and 5 µl Tetramethylethylenediamine (TEMED) (Sigma-Aldrich, UK), and sealed using 1 ml of isopropanol (Sigma-Aldrich, UK). 4% (w/v) polyacrylamide stacking gels were prepared with 6.3 ml of distilled water, 2.5 ml 0.5 M TrisHCl, pH 6.8, 100 µl of 10% SDS (w/v), 1 ml 40% (w/v) acrylamide/bis-acrylamide, 100 µl of 10% (w/v) APS and 10µl TEMED. This

was added to the polymerized resolving gel and a comb was inserted. Once polymerized the protein gel was assembled in running apparatus, placed into a gel tank and covered with 1x running buffer. 5 x running buffer was prepared using: 124 mM Tris hydroxymethyl (methylamine) base (Tris) (ForMedium, UK), 960 mM Glycine (Melford, UK) and 17 mM SDS. Protein samples were boiled in 1 x Protein Sample buffer (Laemmli, Sigma-Aldrich, UK) for 5 min, loaded into wells and electrophoresed at 100V for 1 h 30 min.

Large protein gels were prepared using a vertical dual gel caster (Hoefer). Resolving gels contained 17.31 ml of distilled water, 10 ml of 1.5 M TrisHCl, 400 µl 10% SDS, 12 ml 40% Acrylamide/Bis-acrylamide (Sigma), 250 µl 10% APS and 40 µl TEMED, and sealed with isopropanol. Stacking gels were prepared with 12.78 ml of distilled water, 5 ml 0.5 M TrisHCl, 200 µl of 10% SDS, 2 ml of 40% acrylamide/bis-acrylamide, 200 µl of 10% APS and 20µl TEMED. This was added to the polymerized resolving gel and a comb was inserted. Once polymerized the protein gel was assembled in a standard dual cooled vertical unit running apparatus (Hoefer), placed into a gel tank and covered with 1 x running buffer. Protein samples were boiled in 1 x Protein Sample buffer for 5 min, loaded into wells and electrophoresed at 50 mA for 4 to 6 h. SDS-PAGE gels were either stained overnight at room temperature with Coomassie Blue (Severn Biotech), and de-stained for 1 d in ddH_20 or transferred to nitrocellulose membranes (Amersham Hybond™ ECL™, GE Healthcare, Slough, UK) for Western analysis.

2.13 Western immunoblot analysis

SDS-PAGE gels were transferred to nitrocellulose membranes (Amersham Hybond™ ECL™, GE Healthcare, Slough, UK) for Western analysis in Towbin transfer buffer (20 mM Tris, 154 mM Glycine and 20% (v/v) Methanol (Fisher Scientific, UK), with a Mini Trans-Blot cell (Bio Rad, Hertfordshire, UK) at 60 V for 1 h 30 min. The membrane was incubated in PBS (Oxoid, Cambridge, UK) containing 8% (w/v) powdered milk (Fluka, Sigma-Aldrich, Dorset, UK), at 4 °C overnight. Membrane was washed 3 times with washing buffer; PBS containing 1% (w/v) powdered milk and 0.05% (v/v) Tween 20 (Sigma-Aldrich, Dorset, UK). The membrane was then incubated for 1 h with primary antibody at the dilutions stated

in each chapter. Membrane was washed 3 times with washing buffer (PBS with 0.05% (v/v) Tween 20 and 1% (w/v) powdered milk), and incubated with secondary antibody for 1 h at the dilutions stated in each section. Membrane was washed as before and visualised by enhanced chemiluminescene (ECL), in equal volumes of ECL solutions 1 and 2. ECL solution 1 contained 1 ml Luminol, (Fluka, UK), 250 mM dimethyl sulfoxide (DMSO) (Sigma-Aldrich, UK), 0.44 ml p-coumaric acid (Sigma-Aldrich, UK) 100 mM TrisHCl, with dH_2O to a final volume of 100 ml. ECL solution 2 contained 64 µl 30% H_2O_2 (Sigma-Aldrich, UK), 100 mM TrisHCl, and ddH_2O to a final volume of 100 ml.

2.14 Isolation of bovine peripheral blood lymphocytes (PBLs)

Blood was obtained from Holstein-Friesian cattle aged 18–36 months via jugular vein puncture. Animals were reared indoors and maintained on a ration of hay and concentrates. PBMC were isolated by density gradient centrifugation using Ficoll Paque PLUS, GE Healthcare, Slough, UK) as described previously (Goddeeris & Morrison, 1988).

2.15 T-cell proliferation assays

Bovine PBLs were adjusted to a concentration of 1×10^6 cells/ml in complete cell culture medium, RPMI 1640 medium (Gibco, Invitrogen, Paisley, UK) supplemented with 10% heat-inactivated FCS (Gibco, Paisley, UK), 100 U/ml penicillin, 100 µg/ml streptomycin, 292 µg/ml L-glutamine (PSG) (Gibco, Paisley, UK) and 50 µM 2-mercaptoethanol (Sigma-Aldrich, UK), and cultured in 96 well tissue culture plates (Nunc) for 96 h at 37 °C in humidified air with 5% CO_2. Cells were stimulated at least in triplicate with protein samples as described in each section. Cells in culture medium alone and stimulated with 50 µg/ml Concanavalin A, were used as negative and positive controls respectively. Proliferation of bovine PBMC was assessed by a [^{3}H]-thymidine incorporation assay. Cells were pulsed with 1 µCi/well [^{3}H]-thymidine (GE Healthcare, Slough, UK) after 72 h, and harvested 18 h later using a Tomtec Mach III M Harvester 96 (Hamden, Connecticut, USA) onto Wallac A filters (Perkin Elmer, MA, USA), using a 96-well plate harvester. A Meltilex A wax scintillant strip (Perkin Elmer, MA, USA)

was melted onto the dried filter and [^{3}H] thymidine incorporation into cellular DNA was determined by scintillation counting using a β radiation counter (Wallac 1450 Microbeta PLUS, PerkinElmer, Beaconsfield, Bucks, UK) and recorded as counts per minute (cpm).

Proliferation of bovine PBMC was also assessed by carboxyfluorescein succinimidyl ester (CFSE) staining. Prior to stimulation, PBMC at 2 x 10^7 cells/ml in Hanks buffered saline solution (HBSS) (Gibco, Invitrogen, Paisley, UK) were incubated with 2 µM CFSE for 10 min at 37 °C (Molecular probes, Invitrogen, Paisley, UK). An equal volume of ice cold FCS was added, cells were centrifuged at 250 x g for 20 min and washed 3 times in culture media. Stained cells were cultured and fluorescence associated cell sorting (FACS) analyses were performed after 96 h on a FACScalibur flow cytometer using CellQuest software for data acquisition.

Chapter 3
Diversity, distribution and expression of bovine *S. aureus* RF122 superantigen genes

3.1 Introduction

S. aureus is known to produce at least 21 distinct SAgs, including SEA to E, SEG to J, SER to T, SElK to Q, U, V, and TSST- 1. In recent years, a multitude of *S. aureus* genomes have been sequenced which has led to the identification of genes encoding novel SAgs (Omoe *et al.*, 2005b). *S. aureus* strain RF122 which belongs to the bovine specific lineage ST151, was the first animal associated isolate to be fully sequenced (Herron-Olson *et al.*, 2007). A comprehensive, genome-wide analysis of the complement of SAgs encoded by a bovine strain of *S. aureus* has not been carried out to date.

The aim of this component of the study was to investigate the diversity, distribution and expression of SAgs among bovine *S. aureus* strains by:

- Identifying the full complement of SAg genes in the genome of bovine isolate, RF122
- Investigating SAg gene expression *in vitro* and during infection

3.2 Materials and Methods

3.2.1 Bacterial strains

S. aureus strains investigated in this study were selected to represent a broad variety of clonal genotypes, with diverse host range and geographical origins (Table 3.1). Media was supplemented where appropriate with 50 μg/ml ampicillin (Sigma-Aldrich, UK).

3.2.2 Phylogenetic analysis of staphylococcal SAg genes

The sequences of characterised staphylococcal SAg genes were obtained from the NCBI Genbank database. SAg allelic variants were identified in the fully sequenced *S. aureus* genomes listed in Table 3.2 by Basic Local Alignment Search Tool (BLASTn) analysis. Nucleotide and translated amino acid sequences of all allelic variants of staphylococcal SAg genes were aligned by ClustalW using MEGA 4.0.1 software (Tamura *et al*., 2007). Evolutionary distances were determined by the Maximum Composite Likelihood method, and Neighbour Joining trees were constructed and a bootstrap test of phylogeny performed.

3.2.3 PCR amplification of putative SAg genes

PCR reactions were carried out as described in General Methods using Taq DNA polymerase (Promega, UK), primers to amplify within the coding sequence of putative SAg genes *selx*, *sely* and *selz* (Table 3.3), and 1 μl gDNA isolated from strains listed in Table 3.1. Amplification of Multi Locus Sequence Type (MLST) gene *glpF* was used as a control for the integrity of each gDNA sample (Enright *et al.*, 2000).

Table 3.1: ***S. aureus*** **strains investigated by PCR to determine distribution of** ***selx, sely*** **and** ***selz***

Strain	Host	ST/ET	Geographic origin	Reference/ Source	*selx*	*sely*	*selz*
434 (C434)	Human	8	UK, 1997	(Enright *et al.*, 2000)	+	+	+
LAC	Human CA-MRSA	8	USA	(Kennedy *et al.*, 2008)	+	-	-
88006	Human CA-MRSA	8	USA	(Kennedy *et al.*, 2008)	+	-	-
88007	Human CA-MRSA	8	USA	(Kennedy *et al.*, 2008)	+	-	-
88008	Human CA-MRSA	8	USA	(Kennedy *et al.*, 2008)	+	-	-
88009	Human CA-MRSA	8	USA	(Kennedy *et al.*, 2008)	+	-	-
88010	Human CA-MRSA	8	USA	(Kennedy *et al.*, 2008)	+	-	-
126 (C126P)	Human	12	UK, 1997	(Enright *et al*., 2000)	+	+	-
383 (H383)	Human	22	UK, 1997	(Enright *et al*., 2000)	+	-	-
03-2372.2 #4	Human MRSA	22	UK	Scottish MRSA reference laboratory (SMRSAL)	+	-	-
07-8898.11 #10	Human MRSA	22	UK	SMRSAL	+	-	-
02-2008.L #23	Human MRSA	36	UK	SMRSAL	-	-	-
MSA1832	Human	30	USA, 1968	(Musser & Selander, 1990)	-	-	-
MSA2389	Human furunculosis	45 (ET39)	Sweden	(Musser & Selander, 1990)	+	-	-
MSA2020	Human SSS	121	France	(Musser & Selander, 1990)	+	-	+
MSA2120	Human	126	Denmark, 1983	(Musser & Selander, 1990)	+	-	+
MSA1601	Human MRSA	ET53	USA, 1980s	(Musser & Kapur, 1992)	+	-	-
MSA3418	Human MRSA	ET89	Australia, 1980s	(Musser & Kapur, 1992)	+	-	-

Strain	**Host**	**ST/ET**	**Geographic origin**	**Reference/ Source**	***selx***	***sely***	***selz***
MSA3400	Human MRSA	ET91	Ireland, 1990	(Musser & Kapur, 1992)	+	-	-
MSA1695	Human SSS	ET93	Japan	(Musser & Selander, 1990)	+	+	-
MSA890	Human	ET93	USA	(Musser & Kapur, 1992)	+	-	-
MSA820	Human MRSA	ET93	USA	(Musser & Kapur, 1992)	+	-	-
MSA2965	Human Sepsis	ET191	Canada, 1983	(Musser & Selander, 1990)	+	-	+
B40	Swine	9	Hong Kong	(Guardabassi *et al.*, 2009)	+	-	+
DS95	Ovine	9slv	Denmark	(Mork *et al.*, 2005)	+	-	+
MSA535	Ovine	ET66	Germany	(Musser & Selander, 1990)	+	-	-
DS13 (St7)	Ovine	133	Italy	(Foschino *et al.*, 2002)	+	-	-
DS83 (891-1)	Ovine	133	Norway	(Mork *et al.*, 2005)	+	-	-
ED133	Ovine	133	France, 1997	(Ben Zakour *et al.*, 2008)	+	-	-
DS102 (6659-2)	Ovine	151slv	Sweden	(Mork *et al.*, 2005)	+	+	+
VET-BZ30	Ovine	750	Brazil, 2003	(Aires-de-Sousa *et al.*, 2007)	+	-	-
DS30 (St153)	Caprine	22	Italy	(Foschino *et al.*, 2002)	+	-	-
DS27 (St125)	Caprine	25	Italy	(Foschino *et al.*, 2002)	+	-	-
VI50895	Caprine	130	Norway	(Jorgensen *et al.*, 2005)	+	-	-
DS74 (1563-4)	Caprine	133	Norway	(Mork *et al.*, 2005)	+	-	-
VI50896	Caprine	481	Norway	(Jorgensen *et al.*, 2005)	+	-	-
DS28 (St152)	Caprine	133	Italy	(Foschino *et al.*, 2002)	+	-	-

Strain	Host	ST/ET	Geographic origin	Reference/ Source	*selx*	*sely*	*selz*
RF103	Bovine	71	Ireland	(Fitzgerald *et al.*, 1997)	+	+	-
CTH108	Bovine	115	USA	(Smith *et al.*, 2005)	+	-	-
951	Bovine	126	USA	(Sischo *et al.*, 1993)	+	-	-
MSA948	Bovine	126	USA	Musser J.M	+	-	-
MSA961	Bovine	126	USA	(Musser & Kapur, 1992)	+	-	-
VI50905	Bovine	130	Norway	(Jorgensen *et al*., 2005)	+	-	-
DS36 (V329)	Bovine	133	Sweden	(Cucarella *et al.*, 2001)	+	-	-
RF287	Bovine	133	Ireland, 1986	Fitzgerald, J.R	+	-	-
VI50901	Bovine	133	Norway	(Jorgensen *et al.*, 2005)	+	-	-
38963	Bovine	151	UK	(Sung *et al*., 2008)	+	+	+
RF113	Bovine	151	Ireland	Fitzgerald, J.R	+	+	+
C123/5/005	Bovine	151	UK, 2003	(Sung *et al.*, 2008)	+	-	+
T2 (2521)	Bovine	NK	Ireland	Symth, C.	+	-	+
T6 (2242)	Bovine	NK	Ireland	Symth, C.	+	-	-
T7 (2242)	Bovine	NK	Ireland	Symth, C.	+	-	-
T12 (2242)	Bovine	NK	Ireland	Symth, C.	+	+	-
T21 (2487)	Bovine	NK	Ireland	Symth, C.	+	+	+
T22 (2487)	Bovine	NK	Ireland	Symth, C.	+	-	-

+ or – indicates presence or absence of putative SAg genes.

Table 3.2: *S. aureus* whole genome sequences examined

Strain	Reference number	Geographical origin	Sequence type (ST)	Reference
MSSA476	NC_002953.3	UK, 1997	1	(Holden *et al*., 2004)
MW2	NC_003923.1	USA, 1998	1	(Baba *et al.*, 2002)
N315	NC_002745.2	Japan, 1982	5	(Kuroda *et al.*, 2001)
Mu50	NC_002758.2	Japan, 1997	5	(Kuroda *et al*., 2001)
Mu3	NC_009782	Japan	5	(Neoh *et al.*, 2008)
ED98	NC_013450.1	UK, 96	5	(Lowder *et al.*, 2009)
FPR3757	NC_007793.1	USA, 2003	8	(Diep *et al.*, 2006)
TCH1516	NC_010079.1	USA	8	(Highlander *et al.*, 2007)
Newman	NC_009641.1	UK, 1950	8	(Baba *et al.*, 2008)
NCTC8325	NC_007795.1	UK, 1960	8	(Gillaspy, 2006)
MRSA252	NC_002952.2	UK, 1997	36	(Holden *et al*., 2004)
JH1	NC_009632.1	USA, 2000	105	(Mwangi *et al.*, 2007)
JH9	NC_009487.1	USA, 2000	105	(Mwangi *et al*., 2007)
ED133	CP001996	France	133	(Guinane *et al*., 2010)
RF122	NC_007622	Ireland, 1993	151	(Herron-Olson *et al*., 2007)
COL	NC_002951.2	Colindale, UK	250	(Gill *et al.*, 2005)

Table 3.3 Primers used in this study

Primer	Forward sequence (5´-3´) [a]	Reverse sequence (5´-3´) [a]
PCR		
selx	GGAATTGGGTTTATTCAGAGAGACC	ACTTGTTCAATGTCATTAACACTTTTCAC
sely	TGGTTACAGTAGCTATTCTTTGTTGG	TTAGTTAAGTGCACTTCTATTTCCGTT
selz	AGCGAAACTATGGTTTTTATTGAC	CTTGTGAGTCACTCAATATGTCGC
RT-PCR		
*egc*A	TCTTGATACGTATTTGACACTTGC	TGCGAATGCCCTACCTGATCCTAA
*egc*B	TCCTCTTCCTTCAACAGGTGGAGA	CATCGTGCTTAAACGGAGAGT
*egc*C	ACCTTCTTGTTGGACACACCATCT	AGTTCGCCTTATGAGACTGGCT
*egc*D	TCACCAGATTTCAGATTCAGGCATCA	GCGCAAGGAGATTGGTGTAGGT
*egc*E	GATCAAATCATTGGGACCGGTTG	TCGTCTGTTGATGTATACGGCCT
*egc*F	ACCCGCTAAAGTAACTCCTCCGTA	TTGTCATGGTGAGCATCAAGTGAAA
*egc*G	GCTACTCCTATTTCTTTAGGTTCGT	AGAGTTGTTACAGTCGCTACACC
qRT-PCR		
16srRNA	TATGGAGGAACACCAGTGGCGAAG	TCATCGTTTACGGCGTGGACTACC
*selx*q	AGCAGACGCGTCAACACACAAA	GGTCTCTCTGAATAAACCCAATTCC
*sely*q	GTTGGAAGCTAGAGCAAGACA	GCCAAGAACCCGTATTGACT
*selz*q	CCTACAATGTACGGACAGTGCTCTAC	GGATCATACTTAACTACTGTGCCACG
*tst*q	TGGTATAGTAGTGGGTCTGACGCT	AGGCTGATGTGCCATCTGTGTTT
*sec*q	AACAGTTCACCATATGAAACAGGT	AGATTGGTCAAACTTATCTCCTGGT
*sell*q	ATCTAACGGCGCGATGTAGGTCCA	CTAAGCGGTGTGATTCTGGTGA
*seg*q	TGCGAATGCCCTACCTGATCCTAA	TCCTCTTCCTTCAACAGGTGGAGA
*seln*q	CATCGTGCTTAAACGGAGAGT	ACCTTCTTGTTGGACACACCATCT
*selu*q	AGTTCGCCTTATGAGACTGGCT	TCACCAGATTTCAGATTCAGGCATCA
*sei*q	GCGCAAGGAGATTGGTGTAGGT	GATCAAATCATTGGGACCGGTTG
*selm*q	TCGTCTGTTGATGTATACGGCCT	ACCCGCTAAAGTAACTCCTCCGTA
*selo*q	TTGTCATGGTGAGCATCAAGTGAAA	GCTACTCCTATTTCTTTAGGTTCGT
*se26*q	AACGCAATGTAGTTATGGTGGTGT	TCTGTTTGATGTCCGTCAATCCAT
Cloning		
*selx*pET	TAGCCATATGTCAACACAAAATTCCTCAA	GCGCGGATCCTCAAACTTGTTCAATGTC
*sely*pET	TAGCCTCGAGAGACACAAAATGATCCAAA	GCGCCTCGAGCTACTTTTTAGTTAAGT

Primer	Forward sequence (5´-3´) [a]	Reverse sequence (5´-3´) [a]
*selz*pET	GCGCCATATGAAAACAACTGGATTGATTA	GCGCGGATCCCTATTTCATATAAATATC
*seg*pET	GCGCCATATGCTACCTGATCCTAA	GCGCGGATCCTTATATATTCAGATTC
*sei*pET	GCGCCTCGAGGATATTGGTGTAGGTA	GCGCCTCGAGTTAGTTACTATCTACATA
*selo*pET	GCGCCATATGAATGAAGAAAATCCTA	GCGCGGATCCTTATGTAAATAAATAAAC
*selu*pET	GCGCCATATGAATAAAGCGAGTGAAT	GCGCGGATCCCTTATTTTTTGGTTAA
T7	AAATTAATACGACTCACTATAGG	GCTAGTTATTGCTCAGCGGT

[a] Restriction sites incorporated are underlined.

3.2.4 Transcriptional analysis of SAg genes

3.2.4.1 RNA extraction from *S. aureus*

Total RNA was extracted from *S. aureus* RF122 exponential (OD_{600} = 0.6) and stationary phase (12 h) cultures using the RNeasy miniprep kit (QIAgen, West Sussex, UK) as described in the manufacturer's instructions except for re-suspension in Tris-EDTA with 100 µg/ml lysostaphin and incubation at 37 °C for 20 min. RNA was treated with Turbo DNase (Ambion Inc, Applied Biosystems (AB) Warrington UK) as described in the manufacturer's protocol. Agarose gel electrophoresis of RNA samples in 2% (w/v) agarose gels was carried out to verify the integrity of the RNA.

3.2.4.2 Reverse transcriptase - PCR (RT-PCR)

0.5 µg mRNA from at least 3 separate total-RNA extractions were reverse-transcribed to cDNA with Superscript III reverse transcriptase (Invitrogen), according to the manufacturer's instructions. RT-PCR reactions were carried out to amplify transcripts A to G in a Biorad thermal cycler using PFU DNA polymerase (Stratagene, Agilent, UK) with 100 ng cDNA and forward and reverse primers spanning adjacent genes of the *egc,* listed in Table 3.3. No template (NTC), no Reverse Transcriptase (no RT) and positive genomic DNA controls were included. The thermal programme was 1 cycle at 95 °C for 3 min, followed by 40 cycles at 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min 30 s.

3.2.4.3 Quantitative Reverse Transcriptase - PCR (qRT-PCR)

qRT-PCR reactions were performed using Superscript III Platinum qRT-PCR kit with SYBR Green Supermix-UDG (Invitrogen) using a Mx3000P light cycler (Strategene, Agilent Technologies UK Ltd. Cheshire). qRT-PCR primers were designed to amplify 150 to 200 bp fragments of each target gene and were optimised by absolute quantification with a titration of primer concentrations ranging from 300 nM to 600 nM in 25 µl reactions containing 1 ng RF122 gDNA (Table 3.3). The thermocycling conditions were 10 min at 95 °C for 1 cycle, 20 s at 95 °C, 20 s at 60 °C and 20 s at 72 °C for 40 cycles. The optimal primer concentration chosen to amplify each SAg gene transcript produced the lowest C_T

value and highest flourescence value, with primer concentration reduced where possible. In each case a standard curve was generated from serial 10-fold dilutions of gDNA. The reaction efficiency was calculated based on the slope of the standard curve and expected exponential increase in the amount of template. Optimal values were between 90 to 110%.

Relative values of transcription of SAg genes were obtained using gene-specific qRT-PCR primers listed in Table 3.3 by comparative quantification to the internal control *16SrRNA*. *16S rRNA* primers have been described elsewhere (Ster *et al.*, 2005). RNA samples were processed in triplicate with no template (NTC), no Reverse Transcriptase (no RT) and gDNA controls. Fluorescence was measured at the end of the annealing phase of each cycle and a threshold value for the fluorescence set by the MxPro qPCR software version 4.1. The reaction cycle at which fluorescence exceeds this threshold was identified as the threshold cycle (C_T) and converted to relative quantity of mRNA. qRT-PCR data was analyzed using MxPro software version 4.1.

3.2.5 Cloning and purification of recombinant SAg proteins

Primers, plasmids and restriction enzymes used in the cloning of SAg genes from RF122 into the pET15b plasmid (Novagen, California, USA) are listed in Table 3.3. Forward primers were designed to amplify within the coding sequence of the gene, immediately after the signal peptide, as predicted by Signal P 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). Reverse primers were designed to overlap the stop codon of the gene. Appropriate restriction enzymes were incorporated to facilitate cloning. PCR reactions were carried out with forward and reverse primers listed in Table 3.3, using Vent polymerase (New England Biolabs, Herts, UK). DNA fragments were cloned into Strataclone vector pSC-B (Stratagene, Agilent, UK) as described in the manufacturer's instructions. pSC-B plasmid DNA containing insert was isolated by miniprep (Qiagen, UK) and digested with the appropriate restriction enzymes (New England Biolabs, Herts, UK), purified using QIAquick gel extraction or PCR purification kits from QIAgen (West Sussex, UK), ligated with T4 DNA ligase as described in the manufacturers' instructions (New England Biolabs, Herts, UK), and transformed into *E.coli* DH5α.

The resulting pET constructs were isolated from DH5α using the QIAprep Spin Miniprep kit (QIAgen, West Sussex, UK) as described in the manufacturers instructions, and transformed into BL21 for induction. BL21 cells containing recombinant plasmids were induced when the cultures reached mid-exponential phase of growth, with 0.05 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (ForMedium Ltd., Norfolk, UK) and grown for 4 h. Cells were recovered by centrifugation at 8000 x g, disrupted by French Press, and His-tagged recombinant proteins were purified by affinity chromatography on a Ni-NTA nickel affinity column (Invitrogen, Paisley, UK) as described in the Novagen manual. Protein purity was assessed by SDS-PAGE as described in General Methods.

3.2.6 Western immunoblot analysis

Recombinant proteins were resolved by SDS-PAGE and either stained overnight at room temperature with Coomassie Blue (Severn Biotech) or transferred to nitrocellulose membranes (Amersham Hybond™ ECL™, GE Healthcare, Slough, UK) for Western analysis as described in General Methods. The membrane was incubated for 1 h with primary antibody, either a 1:2500 or 1:5000 dilution of bovine and ovine or human patient serum respectively. The membrane was then incubated with secondary antibodies for 1 h at dilutions of 1:2500 goat anti-bovine IgG/HRP and rabbit anti-sheep IgG/HRP (horseradish perixodase conjugated), (Santa Cruz Biotechnology, Heidelberg, Germany), or 1:5000 polyclonal rabbit anti-human IgG/HRP, Dako, Cambridgeshire, UK. Human sera samples were obtained from infectious endocarditis patients at the New Royal Infirmary, Edinburgh between 2006 and 2009. Sera samples from experimental infection of sheep were provided by E. Vautor (Le Maréchal C, 2009). Sera samples obtained from cows with bovine mastitis were provided by C. Smyth, and were originally obtained from Teagasc Moorepark Dairy Production Research Centre, Fermoy, Co. Cork.

3.3 Results

3.3.1 Bovine *S. aureus* strain RF122 encodes an array of SAg genes

Bioinformatic interrogation of the genome of the sequenced bovine isolate of *S. aureus,* RF122 (Genbank accession number AJ938182) revealed a total of 11 SAg or putative SAg genes and 2 SAg pseudogenes (Table 3.4). The relative location of SAg genes within the genome is indicated in Figure 3.1. There are three bovine-specific allelic variants of characterised SAg genes, *tstbov*, *sellbov* and *secbov* located on the bovine staphylococcal pathogenicity island (SaPIbov), and an *egc* encoding a further 5 bovine specific allelic variants of SAg genes *seg*, *sei*, *selo*, *seln*, *selu* and a pseudogene of *selm*, located on vSaβ. A further 3 genes were identified, SAB0321, SAB0026 and SAB2421c which display significant homology with the nucleotide sequence of previously characterised SAgs (Table 3.4).

SAB0026 shares 98% nucleotide identity with a putative SAg identified in the capsule type 1- associated cassette chromosome element SCCcap1 of *S. aureus* strain M (Luong *et al.*, 2002). Of note, in RF122, SAB0026 is located beside *orfx* (SAB0024), the integration site of staphylococcal cassette chromosome elements, but there is no SCCcap1 element present (Herron-Olson *et al*., 2007). SAB2421c shares 58% nucleotide homology with *set* which encodes the newly described enterotoxin SET (Ono *et al*., 2008), and 53% nucleotide identity with pyrogenic exotoxin G (SPEG) of *S. pyogenes*. SAB0321 encodes a protein annotated as hypothetical in the RF122 genome, which shares 45% nucleotide homology with both *tst* and *ssl7*, and SAB1473c is a pseudogene of a putative SAg gene found in strain Newman (NWMN_1503). The predicted amino acid sequence of SAB1473c reveals the derived protein is truncated after 125 amino acids whereas the full length protein in strain Newman is 235 amino acids long. These putative novel SAg genes have been provisionally named *selxbov* (SAB0321), *selybov* (SAB0026), *selzbov* (SAB2421c) and *se26* (NWMN_1503) according to the standard nomenclature scheme (Lina *et al.*, 2004).

Table 3.4: SAgs encoded by *S. aureus* strain RF122

Gene	Toxin	Locus tag	Homology with characterised SAg gene (%)
tstbov	Toxic shock syndrome toxin-1 ($TSST\text{-}1_{bov}$)	SAB0360c	98% (*tst*)
secbov	Staphylococcal enterotoxin C-bovine (SEC_{bov})	SAB0363	99% (*sec1*)
sellbov	Staphylococcal enterotoxin like toxin L-bovine ($SElL_{bov}$)	SAB0364	99% (*sel1*)
segbov	Staphylococcal enterotoxin G-bovine (SEG_{bov})	SAB1696c	77% (*seg1*)
seibov	Staphylococcal enterotoxin I-bovine (SEI_{bov})	SAB1699c	97% (*sei1*)
selnbov	Staphylococcal enterotoxin like toxin N-bovine ($SElN_{bov}$)	SAB1697c	95% (*sen1*)
selubov	Staphylococcal enterotoxin like toxin U-bovine ($SElU_{bov}$)	SAB1698c	97% (*selu1*)
selmbov	Staphylococcal enterotoxin like toxin M-bovine ($SElM_{bov}$)	SAB1700c	87% (*selm1*)
selobov	Staphylococcal enterotoxin like toxin O-bovine ($SElO_{bov)}$	SAB1701c	98% (*selo2*)
selxbov	Staphylococcal enterotoxin like toxin X-bovine ($SElX_{bov}$)	SAB0321	45% (*tst*)
selybov	Staphylococcal enterotoxin like toxin Y-bovine (SElY)	SAB0026	57% (*seg1*)
selzbov	Staphylococcal enterotoxin like toxin Z-bovine (SElZ)	SAB2421	58% (*set*)
se26	Staphylococcal enterotoxin like toxin 26 (SE26)	SAB1473c	54% (*sea1*)

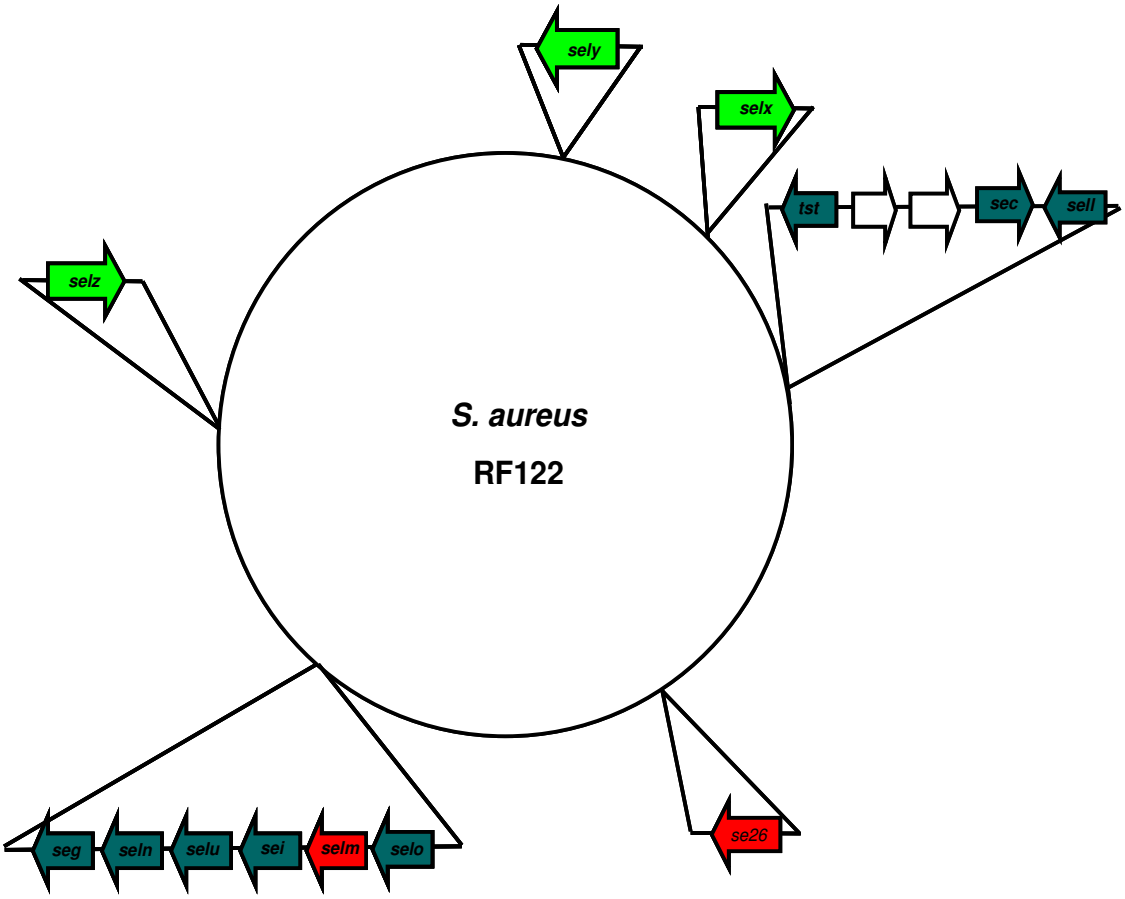


Figure 3.1: Relative positions of SAg genes in the *S. aureus* RF122 genome. Dark green arrows indicate bovine variants of characterised SAg genes, light green arrows denote putative SAg genes, and red arrows represent SAg pseudogenes. Unfilled arrows indicate genes encoding pathogenicity island proteins.

3.3.2 Phylogenetic analysis of SAg genes

Staphylococcal SAg genes are known to display significant sequence homology and can be divided into 4 distinct groups according to their phylogenetic relatedness (Thomas *et al*., 2007, Orwin *et al*., 2002). Sequences of all characterised and putative SAg genes including allelic variants from sequenced *S. aureus* strains available in Genbank (Table 3.2), were identified by BLAST. Phylogenetic analysis revealed that *selx* belongs to Group IV, *sely* to group I, and *selz* to group II (Figure 3.2).

3.3.3 Distribution of putative SAg genes in *S. aureus* strains from humans and animals

Investigation into the distribution of novel SAg genes *selx, sely* and *selz* was carried out by PCR analysis of 57 isolates from a wide range of hosts and geographical origins (Table 3.1). *selx* was found in an unusually high number of strains investigated, including all animal associated strains, 20 bovine, 7 ovine, 6 caprine and 1 swine strain (Table 3.1). *selx* was also present in 21 human isolates and absent in 2 which belong to CC30. Overall 55 of 57 (96.5%) *S. aureus* strains investigated possessed the *selx* gene. In contrast, *sely* was found in only 9 of 57 (15.7%) strains including 3 human, 1 ovine and 5 bovine isolates. Similarly *selzbov* was detected in 11 (19.2%) strains, including 4 human, 2 ovine and 5 bovine isolates (Table 3.1).

3.3.4 SAg genes are expressed at different levels in a growth phase dependent manner

Expression levels of the putative and characterised SAg genes and pseudogenes of *S. aureus* RF122 in mid-exponential and stationary phase cultures were determined by qRT-PCR. Relative quantification of the level of transcription of all 11 genes and 2 pseudogenes, compared to an internal control, *16S rRNA* was carried out. The results indicate that all 11 genes and 2 pseudogenes are transcribed in stationary and mid-exponential phase, with the highest level of transcription detected for the *secbov* gene (Figure 3.3). Overall, SAg genes located on SaPIbov were found to be transcribed at higher levels than the *egc* and putative SAg genes and the SAg

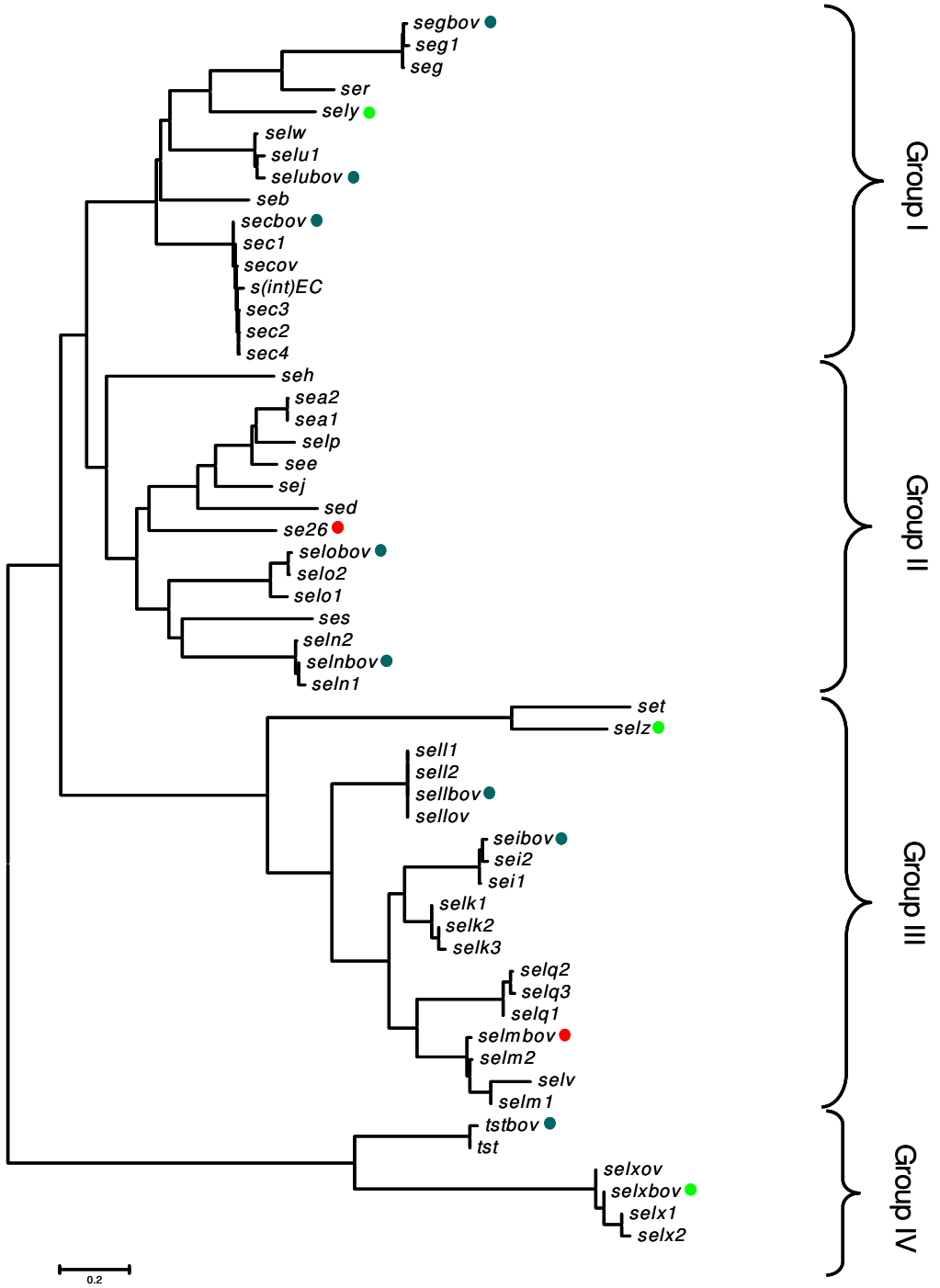


Figure 3.2: Phylogenetic analysis of all characterised staphylococcal SAg genes, including allelic variants from all fully sequenced genomes of *S. aureus*. Circles indicate SAgs encoded by RF122: dark green circles represent bovine variants of characterised SAg genes, light green circles denote putative SAgs and red circles denote pseudogenes.

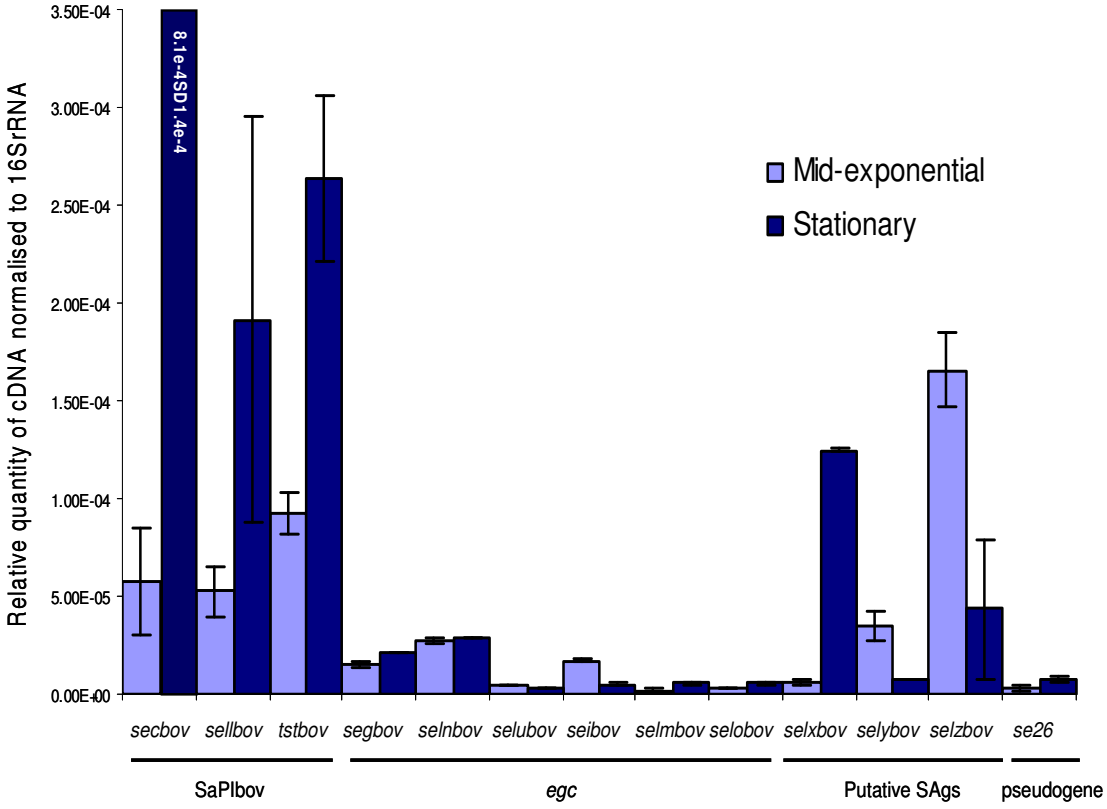


Figure 3.3: Transcription levels of RF122-encoded SAg genes from exponential and stationary cultures, relative to 16S rRNA. Relative quantities of RF122 reverse-transcribed mRNA normalized to the internal control *16SrRNA*, determined by qRT-PCR. Results shown are the means of triplicate experiments and error bars indicate ± S.D

pseudogenes. The results also indicate that SaPIbov SAg genes and putative SAg gene, *selxbov* are upregulated in stationary phase, consistent with regulatory control by *agr*. In contrast, putative SAg genes *sely* and *selz* are transcribed maximally in mid-exponential phase implying a distinct regulatory control. Low level transcription of *egc* genes was detected which was independent of growth phase.

3.3.5 Transcription of *egc* genes

The *egc* genes of human *S. aureus* strain A900322 were previously demonstrated to belong to an operon by RT-PCR analysis (Jarraud *et al.*, 2001). To determine whether the *egc* genes of RF122 are co-transcribed in a similar way, RNA was extracted from RF122 stationary and exponential phase cultures, converted to cDNA by reverse transcription, and amplified by PCR using primer pairs spanning adjacent genes as outlined in Figure 3.4. RT-PCR products of the expected size were obtained for transcripts B (*seg* and *seln*), D (*selu* and *sei*) and F (*selm* and *selo*) in mid-exponential phase and C (*seln* and *selu*) in both mid-exponential and stationary phase indicating co-transcription of these genes (Figure 3.4). In contrast, no RT-PCR products were obtained for the regions A and G from either phase of growth or B, D, E and F in stationary phase. The results indicate that in mid-exponential phase there are two major transcripts, specific for *seg, seln, selu* and *sei*, and *selm* and *selo,* respectively. No transcript was obtained for the region spanning *sei* and the truncated form of *selm.* Northern blot experiments would be useful to further confirm the size of the transcripts.

3.3.6 Recombinant expression of SAgs encoded by RF122

In this study, putative novel SAg genes have been tentatively designated as *selxbov*, *selybov,* and *selzbov*. However to fulfil the standardised toxin nomenclature criteria, expression of the genes must be confirmed, and characterisation of the proteins carried out (Lina., 2004). To confirm that *selxbov*, *selybov, selzbov* encode an intact protein*,* they were cloned into pET15b, and recombinant proteins $rSElX_{bov}$, $rSElY_{bov}$, $rSElZ_{bov}$, proteins were expressed in *E. coli* and purified with the His-tag by Ni^{2+}chelation chromatography. To further investigate the expression of SAg genes encoded by the *egc* which are transcribed by RF122 at low

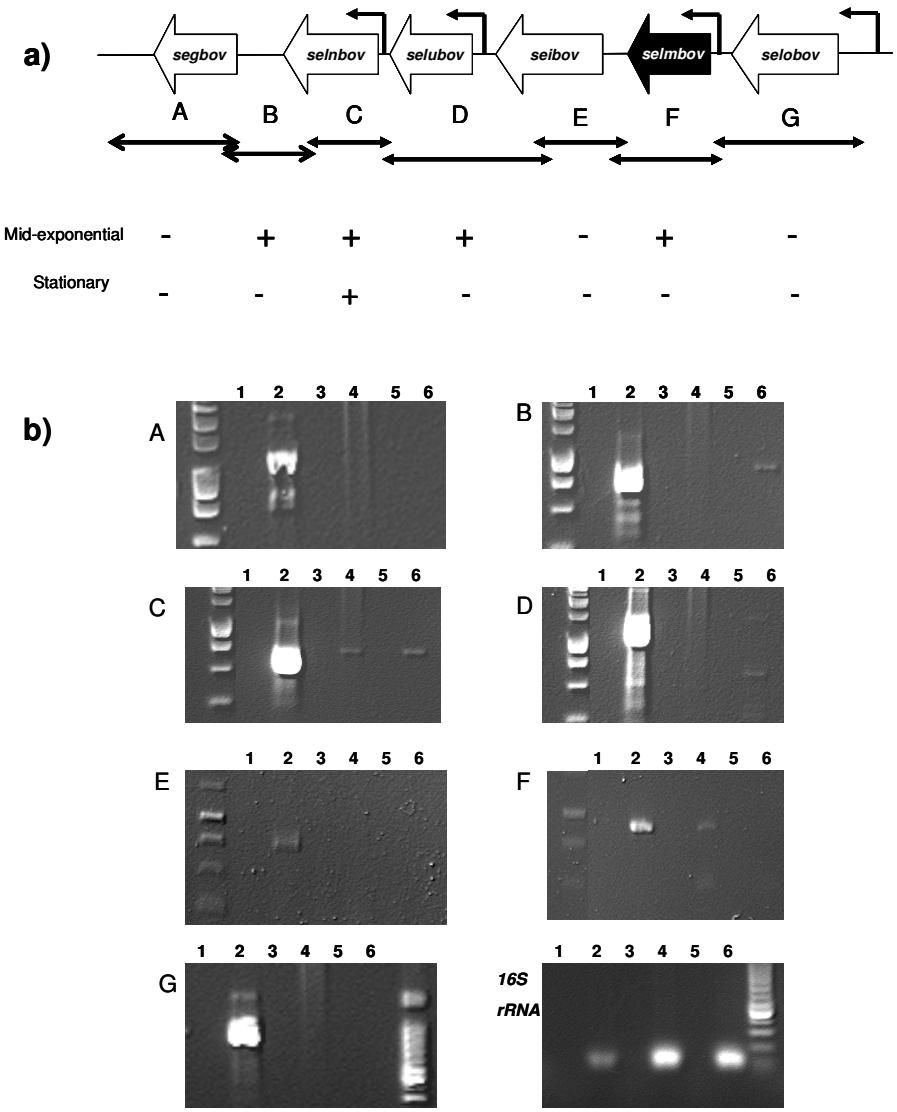


Figure 3.4: Transcriptional architecture of *egc* genes a) Schematic diagram of the order and complement of genes within the *egc* of *S. aureus* RF122. White arrows indicate SAg genes and black arrows indicate pseudogenes. Analysis of *egc* transcripts was carried out by RT-PCR using cDNA prepared from *S. aureus* RF122 total RNA and subjected to PCR using the schematically represented primer pairs A to G. + and – symbols indicate whether or not a transcript was obtained. **b)** RT-PCR analysis of *egc* transcription was carried out using primer pairs A to G. Lane 1, non-template control (NTC); Lane 2, gDNA; Lane 3, noRT stationary phase; Lane 4, stationary phase cDNA; Lane 5, noRT mid-exponential phase; Lane 6, mid-exponential phase cDNA.

levels *in vitro* (Figure 3.3), *segbov, selubov*, *seibov* and *seobov* were also cloned into pET15b and recombinant proteins $rSEG_{bov,}$ $rSEI_{bov,}$ $rSElO_{bov}$ and $rSElU_{bov}$ were purified. Each of the recombinant proteins resolved at their predicted molecular weight on a 10% SDS-PAGE gel, except for $rSEG_{bov,}$ which has a predicted molecular weight of 20.635 kDa but has been shown to resolve at around 23kDa, and $rSEI_{bov}$ which is approximately 27 kDa, larger than the predicted size of 24.6 kDa (Figure 3.5).

3.3.7 Immunogenicity of recombinant SAg proteins with sera from bovine, ovine and human *S. aureus* infections.

To determine whether $SElX_{bov}$, $SElY_{bov}$, $SElZ_{bov}$, $SEG_{bov,}$ $SEI_{bov,}$ $SElO_{bov}$ and $SElU_{bov}$ are expressed *in vivo*, Western immunoblot analysis with convalescent sera was carried out (Table 3.5). Sera sample 2211 obtained from a cow without a history of *S. aureus* mastitis, was included as a negative control and did not contain antibody reactive for any of the SAgs tested (Table 3.5). $rSElX_{bov}$ was reactive with all convalescent sera samples tested, including 5 of ovine, 4 of bovine and 5 of human origin, respectively indicating that an IgG response was generated against SElX during human, bovine and ovine *S. aureus* infections, consistent with an important role in pathogenesis. An IgG response was detected against $rSElY_{bov}$ with all 4 bovine, 2 of 5 human and 1 of 5 ovine sera samples, and rSElZ was reactive with 2 of 5 bovine, 2 of 5 human and 1 of 5 ovine sera samples. IgG responses against *egc*-encoded SAgs were detected in a smaller number of samples, $rSElU_{bov}$ reacted with 2 of 5 bovine and 1 of 5 ovine samples. $rSEI_{bov}$ was reactive with only one human sera sample, and $rSEG_{bov}$ and $rSElO_{bov}$ were not reactive with any of the samples tested. For bovine sera samples 2480 and 2487, the matching *S. aureus* isolates were obtained and screened for the presence of putative SAgs *selx*, *sely* and *selz* (Table 3.1). Serum from cow 2480 contained antibodies against $rSElX_{bov}$ and $rSElY_{bov}$ and was obtained from a cow infected with strain T12 which harbours the encoding genes *selx* and *sely*. Serum sample 2487 contained antibodies specific for $rSElX_{bov}$, $rSElY_{bov}$ and $rSElZ_{bov}$ and was obtained from a cow infected with strain T21, which has the genes encoding for all 3 putative SAgs.

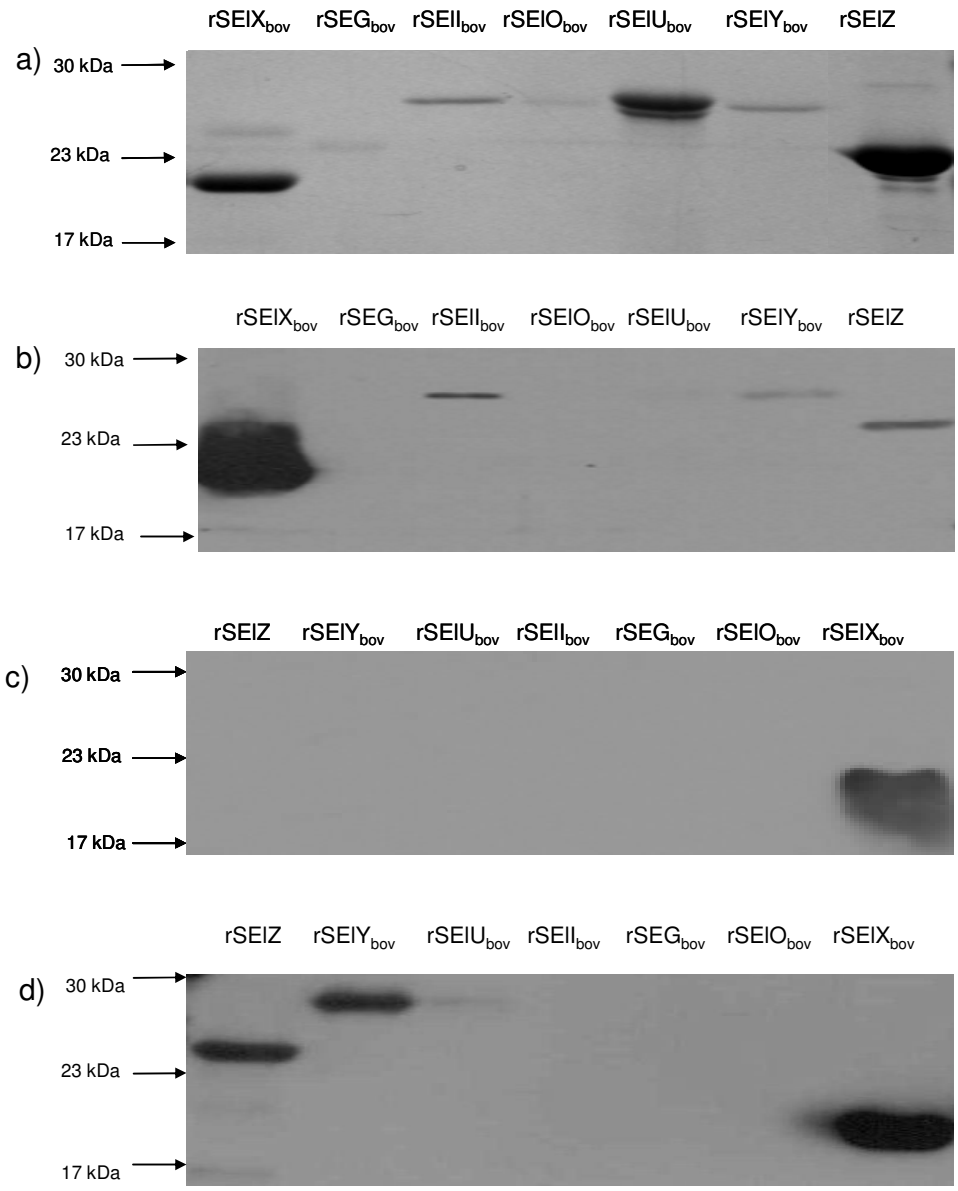


Figure 3.5: **Recombinant expression and immunogenicity of SAgs encoded by RF122 with sera from bovine, ovine and human *S. aureus* infections.** Recombinant SAg proteins were resolved on an SDS-PAGE gel and either stained with Coomassie **a)** or detected by Western blot analysis of the immunogenicity of recombinant SAgs with representative **b)** human, **c)** ovine and **d)** bovine convalescent sera samples.

Table 3.5: Immunogenicity of recombinant SAg proteins with sera from bovine, ovine and human *S. aureus* infections.

Serum sample [a]	rSElX	rSElY	rSElZ	$rSEG_{bov}$	$rSEI_{bov}$	$rSElO_{bov}$	$rSElU_{bov}$
Human							
IE19	+	+	+	-	-	-	-
IE37	+	+	+	-	+	-	-
IE41	+	-	-	-	-	-	-
IE51	+	-	-	-	-	-	-
IE54	+	-	-	-	-	-	-
Ovine							
011	+	+	+	-	-	-	+
040	+	-	-	-	-	-	-
046	+	-	-	-	-	-	-
0112	+	-	-	-	-	-	-
0348	+	-	-	-	-	-	-
Bovine							
2480	+	+	-	-	-	-	-
2487	+	+	+	-	-	-	-
2521	+	+	+	-	-	-	+
4227	+	+	-	-	-	-	+
2211	-	-	-	-	-	-	-

+ or – indicate whether or not serum samples are reactive with SAg proteins.

[a] Human serum samples were obtained from infective endocarditis patients between 2006-2009, New Royal Infirmary of Edinburgh. Serum samples from experimental infection of sheep were provided by E. Vautor (Le Maréchal C, 2009); samples obtained from bovine mastitis, and from Cow 2211 without a history of *S. aureus* infection were provided by C. Smyth, originally obtained from Teagasc Dairy Production Centre in Moorepark, Fermoy, Co. Cork.

3.4 Discussion

Our understanding of the role of SAgs in the pathogenesis of bovine mastitis is very limited. However it has been proposed that they contribute to evasion of the immune response and enhanced persistence within the host (Ferens & Bohach, 2000). We have identified genes encoding 8 previously characterised, 3 novel putative SAgs, and 2 SAg pseudogenes in the sequenced genome of the bovine *S. aureus* strain, RF122. These genes include those located on SaPIbov; *secbov*, *sell* and *tst*, and the *egc* genes; *sei*, *selo*, *seln*, *selu* and truncated forms of *seg* and *selm*. In addition 3 further ORFs displaying significant homology to known SAgs have been identified, SAB0321, SAB0026 and SAB2421c which have provisionally been designated *selxbov*, *selybov* and *selzbov*. The novel SAgs have been named alphabetically in the order of discovery, which is in accordance with the regulations proposed by the International Nomenclature Committee for Staphylococcal Superantigens (Lina *et al*., 2004). Previously, *selw* which is encoded by the *egc* has been described, but to our knowledge SElW has not yet been characterised as a distinct SAg (Collery & Smyth, 2007). Our data indicate that RF122 has the capacity to produce a larger number and wider range of SAgs than was previously appreciated (Fitzgerald *et al*., 2001a).

All SAgs identified to date are encoded by mobile genetic elements (MGE) (Fitzgerald et al., 2001a, Jarraud *et al.*, 2001, Johns & Khan, 1988, Ono *et al.*, 2008, Lindsay & Holden, 2006, Ben Zakour *et al.*, 2008). Therefore, the distribution of SAg genes among *S. aureus* strains is highly variable. Omoe *et al*, showed that 80% of human nasal isolates contain at least one SAg gene, including 50% which contain the *egc* locus and Smyth *et al* demonstrated that 57% of animal associated strains screened contained at least one SAg gene with the *egc* found in 30% of isolates (Smyth *et al.*, 2005, Omoe *et al.*, 2005a). These studies suggest that no single SAg is encoded by more than 50% of strains and that about a fifth of all strains may not have the capacity to produce SAgs at all. In this study the distribution of putative novel SAgs *selx, sely* and *selz* in a diverse range of *S. aureus* strains from a wide range of hosts was investigated (Table 3.1). *selx* is the most prevalent SAg gene, found in all isolates examined except for isolates belonging to the CC30 lineage

(96.5% of all *S. aureus* strains investigated). In contrast *sely* and *selz* were identified in a much lower percentage of strains, 15.5% and 18.6% respectively.

Allelic variation of staphylococcal SAgs genes including *tst, sec, seg* and *sei* has been reported previously (Fernandez et al., 2006a, Jarraud *et al.*, 2001, Thomas *et al.*, 2006, Ho *et al.*, 1989). In this study, phylogenetic comparison of novel SAgs to previously identified SAg genes revealed that *sely* belongs to group I, *selz* to group II and *selx* to Group IV (Figure 3.2). Previously the only member of the most phylogenetically distinct group IV was *tst* (Thomas *et al*., 2007). It is noteworthy that RF122 has the potential to encode SAgs belonging to each of the phylogenetic groups, which could potentially lead to the activation of a wide array of Vβ subfamilies (Figure 3.2). Additionally, individual members of SAg gene clusters such as SaPIbov and *egc* SAgs belong to 3 distinct phylogenetic groups, which suggests an advantage for the bacteria to encode phylogenetically distinct SAgs.

Relative expression levels of RF122-encoded SAg genes in stationary and mid-exponential phase were determined by qRT-PCR, and revealed that all characterised and putative SAg genes and pseudogenes are transcribed in both phases of growth. High levels of expression of SaPIbov genes, and in particular *secbov* were detected. The *egc* genes were transcribed at very low levels *in vitro.* The growth phase-dependent upregulation of genes encoding SaPIbov SAgs and the putative SAg, $SElX_{bov}$ in stationary phase cultures is consistent with *agr* control. *agr* control of expression has previously been determined for SEB, SEC and SED (Gaskill & Khan, 1988, Tseng *et al.*, 2004, Regassa *et al.*, 1991, Derzelle *et al.*, 2009). In the current study, transcription of putative SAg genes *sely* and *selz* was also shown to be growth phase-dependent with upregulation in mid-exponential phase. In this study transcription of *egc* genes has not been shown to be growth phase-dependent; in contrast to a previous study which described upregulation of *egc* genes in exponential phase (Grumann *et al.*, 2008). Our results are consistent with those of Derzelle *et al*, who have described a low abundance of *egc* transcripts produced by *S. aureus* strains and only a slight decrease in SAg gene transcription in post-exponential phase (Derzelle *et al.*, 2009). These results imply that SAgs encoded by the *egc* may be less important than other SAgs in *S. aureus* pathogenesis. However, these *in vitro* results may not reflect the situation *in vivo*.

Previously, Northern blot analysis revealed *seg1* and *sei1* were located on an unusually large transcript which suggested *egc* genes could be transcribed on a polycistronic locus (Munson *et al.*, 1998a). *egc* genes of human isolate A900322 were demonstrated to belong to an operon, by RT-PCR analysis (Jarraud *et al*., 2001, Munson *et al*., 1998a). RT-PCR analysis of the *egc* region of RF122 in the current study indicates that *egc* genes *segbov*, *selnbov*, *selubov* and *seibov* are co-transcribed, as are *selm* and *selobov* in mid-exponential phase. *selnbov* and *selubov* which are in close proximity are co-transcribed in both mid-exponential and stationary phase of growth. There is no transcript detected between *seibov* and the truncated form of *selm* in either phase of growth. These results indicate transcription of the *egc* in RF122 is distinct from that determined for the human strain, A900322 in the initial study (Jarraud *et al*., 2001). Sequence variation in the egc is commonly observed between strains (Letertre *et al*., 2003). The archetypal egc cluster identified in A900322 contains 5 SAg genes *segL29P*, *seln1*, *sei1, selm1 selo1* and 2 pseudogenes φent1 and φent2. In contrast, the *egc* of RF122 encodes *selnbov*, *selubov*, *seibov*, *selobov*, and truncated forms of *selm* and *seg* (Figure 3.4). Strain-dependent sequence variation of the *egc* between strains is likely to be responsible for variable transcription. Putative -35 and -10 promoter sequences, TTGTCT and TAATTT respectively were identified by Jarraud *et al*, upstream of the *selo1* gene (Jarraud *et al.*, 2001). At the same genomic location in RF122 the -35 sequence is conserved, but the -10 sequence is unique, TAGTTT. However this region has been predicted as a putative promoter site, using NNPP / Prokaryotic, a prokaryotic promoter prediction tool (Bayer College of Medicine). In addition there were a number of other putative -35 and -10 promoter sequences identified in the RF122 egc locus (data not shown), which are consistent with the transcriptional architecture predicted in the current study (Figure 3.4).

Most human adults have antibodies specific for *S. aureus* SAgs including SEA, SEB, SEC, SED, SEE and TSST-1, presumably as a result of exposure to *S. aureus* during colonisation or infection (Takei *et al.*, 1993, Ulrich, 2000). Despite the relatively high prevalence of the *egc* cluster in clinical isolates of *S. aureus*, neutralising antibodies are rare (Holtfreter *et al.*, 2004). Importantly in this study we have shown that SElX, SElY, SElZ, and to a lesser extent SElU and SEI are expressed by *S. aureus in vivo.* Antibodies against each of the putative SAgs SElX,

SElY and SElZ have been detected in at least one serum sample of bovine, ovine and human origin, which suggests they are important in a range of hosts. In particular, an IgG response against SElX was detected in all sera samples examined. Taken together with the unusually high prevalence of the encoding gene, these data suggest an important role in staphylococcal disease pathogenesis. The results of this study further confirm that antibodies against *egc* genes are not frequently detected, implying a limited role for *egc*-encoded SAgs in human and animal infections.

Chapter 4
Functional evolutionary characterisation of a *Staphylococcus aureus* core genome-encoded superantigen

4.1 Introduction

In this study we have described the discovery of a putative novel SAg gene *selxbov* in the genome of the bovine *S. aureus* strain, RF122, and have demonstrated expression of the putative novel SAg *in vitro* and *in vivo.* Remarkably, we have discovered that the *selx* gene was present in 96.5% of diverse *S. aureus* strains examined, including human and ruminant strains, implying a broad role in pathogenesis in multiple host species. However designation as a SAg requires further experiments to fulfil the criteria of the International Nomenclature Committee for Staphylococcal Superantigens, including Vβ specific T-cell activation, pyrogenicity, lethality to rabbits in miniosmotic pumps and endotoxin enhancement (Lina *et al*., 2004).

In this study the aim was to further investigate the function and molecular origin of this novel SAg, by:

- Examining the molecular evolution of *selx*

- Functional characterisation of SElX by examination of bovine T-cell activation capacity

4.2 Materials and Methods

4.2.1 Bacterial strains and plasmids

S. aureus strains used in this study are described in Table 4.1. *E. coli* strains BL21 and DH5α were used as cloning hosts. Media supplemented where appropriate with 50 μg/ml ampicillin, and 150 μg/ml X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside).

4.2.2 DNA sequencing of *selx*

gDNA was extracted from strains indicated in Table 4.1 as described in General Methods. PCR amplification of *selx* was carried out with PfuUltra II Fusion DNA Polymerase (Agilent, UK), using 100 nM forward and reverse primers; *selx*seq-F: TGGTAGCAAATTAAAGTTAATCAAGAG and *selx*seq-R: TGCTAATCATAACAAAGAAAGCTAGG, as described in General Methods. PCR products were purified using a QIAquick purification kit and sequenced by Genepool Sequencing Service (King's Buildings, University of Edinburgh, UK). PCR amplification of *selx* for DNA sequencing was carried out by R. Cartwright, University of Edinburgh, UK.

4.2.3 Phylogenetic analysis

The sequences of *selx* alleles were obtained from DNA sequencing and genome sequence files deposited in GenBank (Table 4.2). Nucleotide and translated amino acid sequences were aligned by ClustalW using MEGA 4.0.1 software as described in General Methods. Recombination detection program RDP v3.39 was used to identify putative end points of recombination in *selx* alleles (Heath *et al.*, 2006). Recombination events predicted were detected by at least 3 of 6 RDP programs ($P<0.05$).

Table 4.1: *S. aureus* strains used in this study

Strain	Host	Geographic origin	MLST/ PFGE	Reference/ Source
Newman$^{\Psi}$	Human	UK	8	(Duthie, 1952)
LAC $^{\Psi}$	Human	USA	8	(Voyich *et al*., 2006)
88008 $^{\Psi}$	Human	USA	8	(Kennedy *et al.*, 2008)
88010 $^{\Psi}$	Human	USA	8	(Kennedy *et al*., 2008)
126*	Human	UK	12	(Enright *et al.*, 2000)
MSA2389*	Human	Sweden	45	(Musser & Selander, 1990)
MSA2020*$^{\Psi}$	Human	France	121	(Musser & Selander, 1990)
MSA2965 $^{\Psi}$	Human	Canada,	ET191	(Musser & Selander, 1990)
RF103*	Bovine	Ireland	71	(Fitzgerald *et al.*, 1997)
RF31*	Bovine	Ireland	97	Fitzgerald J.R
951*	Bovine	USA	126	(Sischo *et al.*, 1993)
RF287 $^{\Psi}$	Bovine	Ireland,	133	Fitzgerald J.R
VI50901 $^{\Psi}$	Bovine	Norway	133	(Jorgensen *et al.*, 2005)
RF122 $^{\Psi}$	Bovine	Ireland,	151	(Fitzgerald *et al*., 1997)
RF122-8	Bovine	Ireland	151	Section 6.3.4
RF113 $^{\Psi}$	Bovine	Ireland	151	Fitzgerald J.R
ED98 $^{\Phi}$	Avian	N. Ireland	5	(Rodgers *et al.*, 1999)
DS27 *	Caprine	Italy	25	(Foschino *et al.*, 2002)
VI50895 $^{\Psi}$	Caprine	Norway	130	(Jorgensen *et al*., 2005)
ED133 $^{\Psi}$	Ovine	France,	133	(Ben Zakour *et al*., 2008)
DS83 $^{\Psi}$	Ovine	Norway	133	(Mork *et al.*, 2005)
DS95 (1) $^{\Psi}$	Ovine	Denmark	706	(Mork *et al*., 2005)
VET-BZ30 $^{\Phi}$	Ovine	Brazil,	750	(Aires-de-Sousa *et al.*, 2007)
B40*	Swine	Hong Kong	9	(Guardabassi *et al.*, 2009)

* DNA sequencing of *selx* gene was carried out

$^{\Psi}$/$^{\Phi}$ SElX production was detected/not detected by Western analysis

Table 4.2: Distribution and coordinates of *selx* in sequenced *S. aureus* genomes.

Strain	Reference number	ST	Presence of *selx*	Locus tag/ coordinates
MSSA476	NC_002953.3	1	+	SAS0347
MW2	NC_003923.1	1	+	MW0345
TCH70	ACHH01000007.1	1	+	12962-12351
N315	NC_002745.2	5	+	SA0357
Mu50	NC_002758.2	5	+	SAV0370
Mu50_omega	BABM01000001.1	5	+	415121-415732
Mu3	NC_009782	5	+	SAHV_0367
CF marsielle	CABA01000104.1	5	+	34300-33689
MR1	ACZQ01000042.1	5	+	21763-21152
ED98	NC_013450.1	5	+	377804-378415
A10102	ACSO01000006.1	5	+	79351-79962
A5937	ACKC01000031.1	5	+	354030-354641
A6224	ACKE01000012.1	5	+	48160-48771
A9781	ACKL01000029.1	5	+	9191-9802
A6300	ACKF01000002.1	5	+	53512-54123
A9299	ACKH01000031.1	5	+	89178-88567
A9719	ACKJ01000043	5	+	261259-261870
A8155	ACKG01000031.1	5	+	261394-262005
A9763	ACKK01000029.1	5	+	89367-88756
A5948	ACKD01000059.1	8	+	35867-35256
TCHFPR3757	NC_007793.1	8	+	SAUSA300_0370
USA300	NC_010079.1	8	+	USA300HOU_039
TCH1516				2
Newman	NC_009641.1	8	+	NWMN_0362
NCTC8325	NC_007795.1	8	+	SAOUHSC_00354
132	ACOT01000013.1	8	+	85792-86403
D30	ABFB01000008.1	8	+	27371-26760
930918-3	ABFA01000030.1	8	+	34590-33979

H19	ACSS01000018.1	10	+	252814-253425
Strain	**Reference number**	**ST**	**Presence of *selx***	**Locus tag/ coordinates**
D139	ACSR01000010.1	10	+	3446-4057
NOH4	Sanger	22 slv	+	NK
EMRSA-15	Sanger	22	+	NK
A9635	ACKI01000032.1	45slv	+	90134-90562
TCH60	ACHC01000008.1	30	-	N/A
MRSA252	NC_002952.2	36	-	N/A
M876	ACJV01000012.1	30	-	N/A
MN8	ACJA01000076.1	30	-	N/A
TCH130	ACHD01000271.1	72	+	27060-27671
JH1	NC_009632.1	105	+	SaurJH1_0429
JH9	NC_009487.1	105	+	SaurJH9_0419
RF122	NC_007622	151	+	SAB0321
058	FN433596.1	239	+	467388- 467999
2/TW20				
JKD6008	ABRZ01000012.1	239	+	5328-5939
JKD6009	ABSA01000060.1	239	+	5327-5938
COL	NC_002951.2	250	+	SACOL0442
USA300 TCH959	AASB02000040.1	1159	+	71002 – 71613

4.2.4 Transcriptional analysis of *selx*.

Total RNA was extracted from *S. aureus* strains RF122, LAC and ED133 exponential (OD_{600} = 0.6) and stationary phase (12 h) cultures using the RNeasy miniprep kit (QIAgen, West Sussex, UK) as described in the manufacturer's instructions except for re-suspension in TE buffer with 100 µg/ml Lysostaphin and incubation at 37 °C for 20 min. RNA was treated with Turbo DNase (Ambion Inc, Applied Biosystems (AB) Warrington UK) as described in the manufacturer's protocol. 0.5 µg mRNA from at least 3 separate total-RNA extractions were reverse-transcribed to cDNA with the Power SYBR® Green RNA-to-CT 2-Step Kit (AB, Warrington, UK) as described in the manufacturer's protocol.

To quantify cDNA generated by reverse transcription from target RNA, qRT-PCR reactions were carried out in 25 µl reactions containing 50 ng of cDNA, 300 nM *selxq* and *16S rRNA* primers (Table 3.3), and SYBR Green I dye master mix (AB, Warrington, UK), in a Stratagene Mx3000P light cycler (Strategene, Agilent Technologies UK Ltd. Cheshire). Relative values of transcription of *selx* was determined by comparative quantification to the internal control *16SrRNA*. The thermal conditions were: 10 min at 95 °C for 1 cycle, 20 s at 95 °C, 20 s at 60 °C and 20 s at 72 °C for 40 cycles. RNA samples were processed in triplicate with no template (NTC), no Reverse Transcriptase (no RT) and positive genomic DNA controls. Fluorescence was measured at the end of the annealing phase of each cycle and a threshold value for the fluorescence set by the MxPro qPCR software version 4.1. The reaction cycle at which fluorescence exceeds this threshold was identified as the threshold cycle (C_T) and converted to relative quantity of mRNA.

4.2.5 Cloning and purification of recombinant SEIX variants.

selx alleles, *selxov* and *selx2* from strains ED133 and Newman respectively, were cloned into the pET15b plasmid as described for *selxbov* in Section 3.2.5, using *selx*pET primers listed in Table 3.3. Protein purity was assessed by SDS-PAGE. Samples were dialysed using Spectra/Por®Float-A-Lyzer tubing with 8 to 10 kDa MWCO (Spectrum Laboratories, Fischer Scientific, Leicestershire, UK) Lipopolysaccharide (LPS) was removed by affinity chromatography with Detoxi

Gel endotoxin removal gel as described in the manufacturer's instructions (Pierce, Thermo Fisher Scientific, Northumberland, UK).

4.2.6 Concentration of secreted *S. aureus* proteins

S. aureus strains selected to represent a diverse host range (Table 4.1) were grown in 10 ml TSB until stationary phase. Cultures were centrifuged at 4000 x g for 20 min and supernatant fractions were concentrated with Amicon Ultra-15 Centrifugal Filter units (10 kDa MWCO) as described in the manufacturers instructions (Millipore, Watford, UK).

4.2.7 Western immunoblot analysis.

Recombinant proteins and concentrated secreted proteins were seperated by SDS-PAGE and transferred to nitrocellulose membranes (Amersham Hybond™ ECL™, GE Healthcare, Slough, UK) as described in General Methods. The membrane was then incubated for 1 h with sera samples diluted 1:2500 (bovine and ovine), or 1:5000 (human) in washing buffer, or 2 h with 1:1500 dilution of rat antisera raised against $rSElX_{bov}$. Membrane was washed and incubated with secondary antibodies for 1 h at dilutions; 1:2500 (Goat anti-bovine IgG/HRP, and rabbit anti-sheep IgG/HRP, Santa Cruz Biotechnology, Heidelberg, Germany), 1:5000 (Polyclonal rabbit anti-human IgG/HRP, Dako, Cambridgeshire, UK), or 1:1500 (Goat polyclonal antibody to rat IgG/HRP, Abcam, Cambridge, UK). Membrane was washed as before and visualised by enhanced chemiluminescene (ECL). Sera samples were obtained as described in Section 3.2.6. Semiquantitative spot densitometry was carried out with the ChemiImager 4000i.V4 program, by use of a MultiImager light cabinet (Alpha Innotech).

4.2.8 Bovine T-cell proliferation assays

Blood was obtained from Holstein–Friesian cattle, animals 1683 and 1693, aged 18 to 36 months via jugular vein venupuncture. Animals were reared indoors and maintained on a ration of hay and concentrates. PBMC were isolated from blood by density gradient centrifugation as described in General Methods, and stimulated with ten-fold dilutions (10 µg/ml to 1 fg/ml) at least in triplicate of rSElX variants. Culture media and 50 µg/ml Concanavalin A were used as negative and positive

controls respectively. Proliferation of Bovine PBMC was assessed by a [^{3}H]-thymidine incorporation assay as described in General Methods. Total RNA was extracted from bovine PBMC (4×10^6 cells) prior to stimulation or after stimulation with rSElX proteins (1 µg/ml) for 96 h, using Tri-reagent (Sigma–Aldrich, Dorset, UK) as described in the suppliers instructions. Vβ-dependent T-cell activation in response to rSElX variants was determined using the qRT-PCR bovVβ assay described in Section 5.2.5.

4.2.9 Protein structural modelling

Translated nucleotide sequences of *selx2, selxbov* and *selxov* were modelled using Phyre (Kelley & Sternberg, 2009). Predicted 3D structures were were generated for $SElX_2$, $SElX_{bov}$ and $SElX_{ov}$ using the crystal structure of TSST-1 as a template, protein data bank (PDB) file, c5tssA, and analysed using Pymol software (DeLano, 2002).

4.3 Results

4.3.1 *selx* is encoded by the majority of *S. aureus* strains

Bioinformatic interrogation of 45 publicly available *S. aureus* genomes for the presence of *selx* was carried out by BLASTx analysis. In addition to the 57 isolates previously examined by PCR (Table 3.1), the strains (n = 102) represent the full breadth of species diversity and include the most important human and animal pathogenic clones. Remarkably, of the 102 isolates, 96 (94%) contained the *selx* gene which included all strains examined except isolates of the CC30 clonal complex (Table 3.1 and Table 4.2). BLASTx analysis also revealed the absence of *selx* in other staphylococcal species. The high prevalence of *selx* in *S. aureus* strains is unique, and indicative of core-genome location.

Analysis of the genomic location of *selx* amongst sequenced strains representing diverse STs (ST1, 5, 8, 10, 45slv, 72, 105, 133, 151, 239, 250 and 1159) (Table 4.2) revealed that *selx* is located at an identical chromosomal site in all strains examined. Specifically, it is located about ~400 kb from the origin of replication in the *oriC* environ among a cluster of 4 genes specific for *S. aureus* including a predicted integrase pseudogene and 2 genes encoding hypothetical proteins of unknown function (Figure 4.1). The cluster is flanked by conserved genes encoding ribosomal proteins and a DNA-binding protein involved in DNA replication (Figure 4.1) which are located at the same genomic location in other staphylococcal species such as *Staphylococcus epidermidis* (Figure 4.1). The genetic linkage of *selx* with an integrase pseudogene, its wide distribution across the full breadth of *S. aureus* species diversity, and its absence in the genome of other staphylococcal species indicates an ancient horizontal acquisition which may have occurred during *S. aureus* speciation. The existence of a single *S. aureus* clonal complex (CC30) which does not contain *selx* but contains the adjacent integrase pseudogene at the same chromosomal site, is consistent with a deletion event in a progenitor of the lineage which contained the *selx* gene (Figure 4.1).

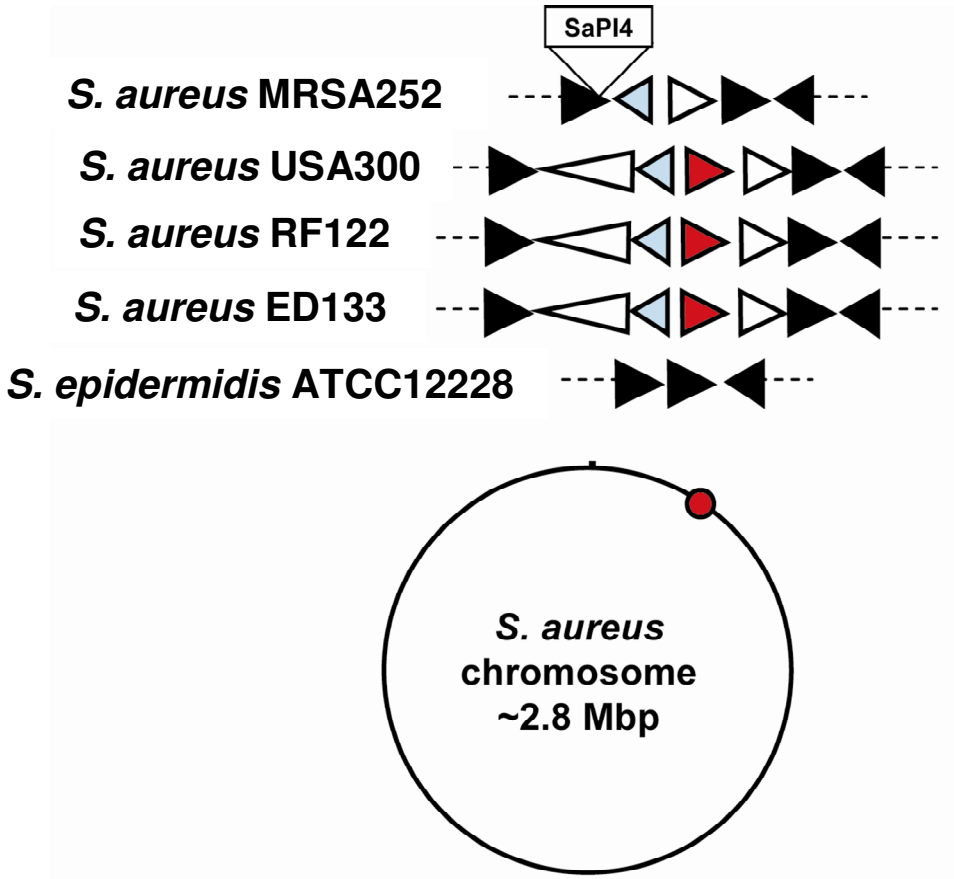
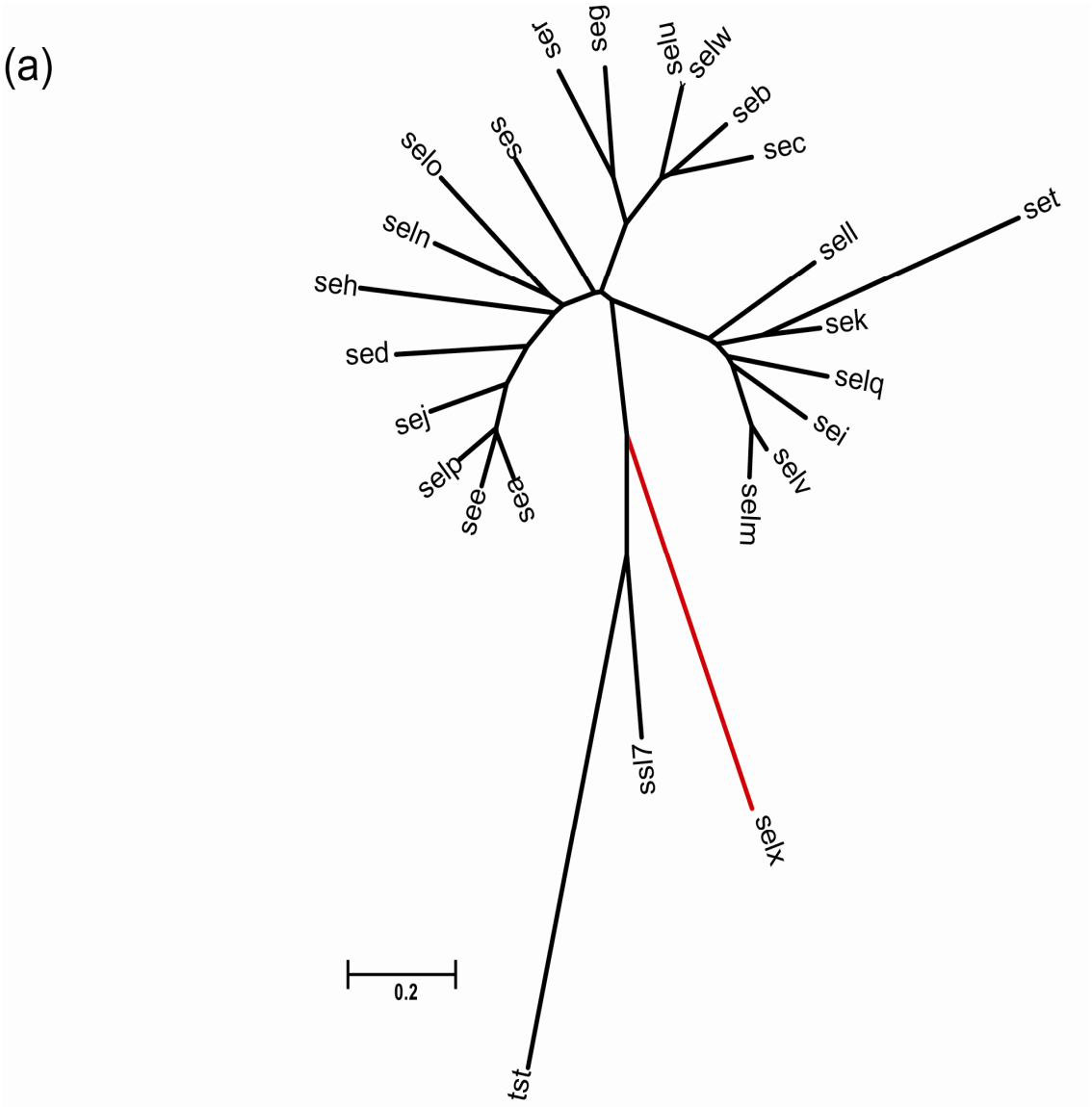


Figure 4.1. Genomic location of *selx* in *S. aureus*. *selx* is depicted by a red arrow, the integrase pseudogene is depicted with a blue arrow, conserved staphylococcal genes conserved genes encoding ribosomal proteins and a DNA-binding protein involved in DNA replication are indicated by black arrows, and white arrows represent hypothetical proteins of unknown function.

4.3.2 *selx* exhibits species-wide allelic variation and evidence for assortive recombination

To examine the allelic variation of *selx* among *S. aureus* strains representing the breadth of species diversity, DNA sequencing of *selx* was carried out for 9 *S. aureus* strains of ST 9, 12, 25, 45 slv, 97, 121 and 126, indicated in Table 4.1 and compared to the sequence of *selx* for 41 strains available in Genbank (Table 4.2). *selx* contained a total of 8.27% variable nucleotide sites and was represented by 14 distinct allelic variants encoded by human and animal strains, designated *selx1-11*, *selxbov1, selxbov2 and selxov,* respectively.

In general, strains belonging to each clonal complex encode a unique *selx* allele (Figure 4.1b) consistent with point mutation being the major driving force for evolution within individual lineages. However, 2 alleles, *selx2* and *selx5* were associated with multiple clonal complexes indicating that assortive recombination of the *selx* gene has contributed to the distribution of the *selx* locus in some strains. Specifically, *selx2* was identified in ST12 and CC8 strains, and *selx5* was encoded by ST1 and ST72 strains, respectively. In addition, examination of the role of recombination in *selx* diversity employing the RDP3 suite of detection programs (Heath *et al*., 2006) revealed evidence for at least 2 distinct recombination events leading to hybrid alleles of *selx* (Figure 4.3). Furthermore, a phylogenetic tree based on *selx* gene sequences has a topology which was very distinct from the phylogenetic tree derived from concatentated MLST loci (Figure 4.2). Taken together, these data suggest that recombination has contributed to the species-wide diversity of *selx*.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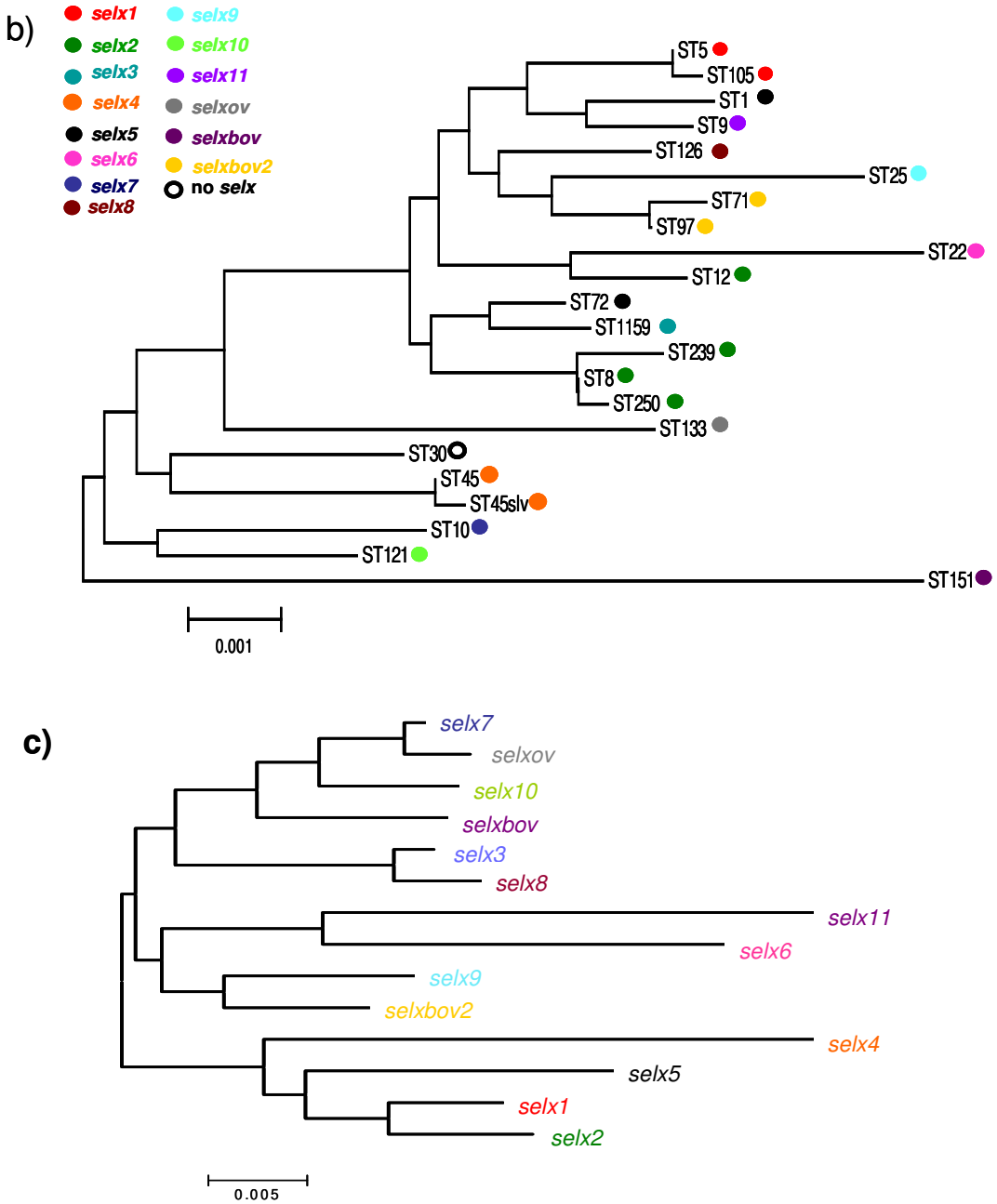

Figure 4.2: Phylogenetic analysis of *selx* and its species-wide distribution. a) Neighbour-joining tree based on all known staphylococcal SAgs and SAg-like protein ssl7 showing their relatedness to *selx*. **b)** Neighbour-joining tree of concatenated MLST sequences of representative *S. aureus* STs representing the breadth of species diversity. Coloured circles denote the presence of specific *selx* alleles. **c)** Neighbour-joining tree of all *selx* alleles identified in this study.

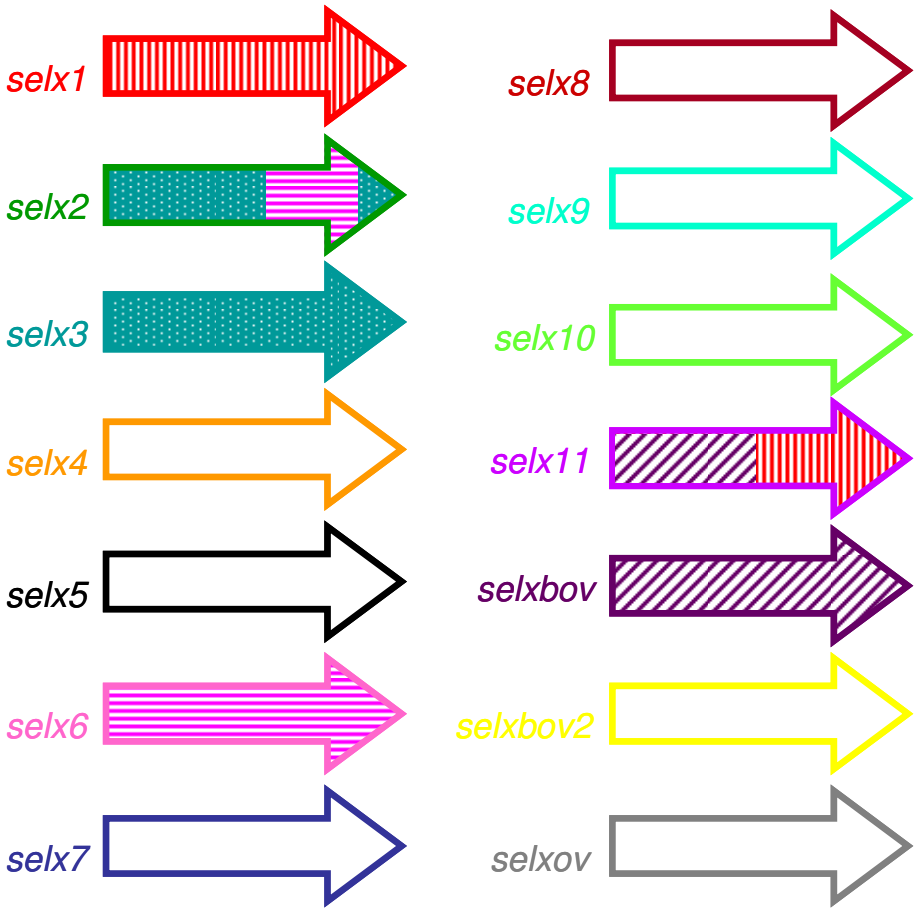

Figure 4.3 Predicted recombination sites identified in *selx* alleles. Recombination events and their breakpoints were detected by RDP v3.39 software and those with significant P value, P<0.05 in at least three of seven recombination detecting programs are indicated. Filled arrows indicate parent and recombinant sequences.

4.3.3 SElX is expressed by clinical isolates *in vitro* and during human, bovine and ovine infection

In Section 3.3.4 we described growth-phase dependent transcription of *selx* by bovine *S. aureus* strain RF122. To investigate whether the transcription of representative clinical isolates of human and ovine origin, LAC and ED133 was similar to RF122, qRT-PCR was carried out. *selx* was transcribed by each strain in a growth-phase dependent manner with highest expression during stationary phase (Figure 4.4). To further investigate the *in vitro* expression of SElX we carried out Western blot analysis with stationary phase culture supernatants of 15 clinical isolates of human, bovine, and ovine origin using rat anti-sera specific for $rSElX_{bov}$ which was generated by Keun Seok Seo at the University of Idaho. Expression of SElX was detected in 14 of 15 isolates in total including 6 of 6 human strains isolated from sepsis, Scalded Skin Syndrome and infective endocarditis patients, 4 of 4 bovine mastitis, 3 of 4 ovine mastitis and 1 caprine mastitis isolate, indicating that SElX is made by most clinical isolates at detectable levels *in vitro* (Figure 4.4 and Table 4.1).

To determine whether SElX is expressed during human and animal colonization or infection, Western immunoblot analysis was carried out with recombinant SElX human, bovine and ovine variants and sera from humans, cows and sheep recovering from *S. aureus* infections. All 5 human, 4 bovine and 5 ovine sera samples contained antibody which cross-reacted with the SElX recombinant proteins but not with recombinant SElO SAg suggesting that SElX is made during *S. aureus* colonization or infection of humans and animals and stimulates a humoral immune response. Of note, densitometric analysis of SElX immunoreactive bands indicated a approximate 2-fold lower affinity of human and bovine IgG for $SElX_{ov}$ in comparison to ovine IgG suggesting that $SElX_{ov}$ has structural differences leading to distinct epitopes which are less reactive with human and bovine IgG (Figure 4.4). The expression of SElX during infection of humans and animals suggests an important general role in *S. aureus* disease pathogenesis in multiple host species.

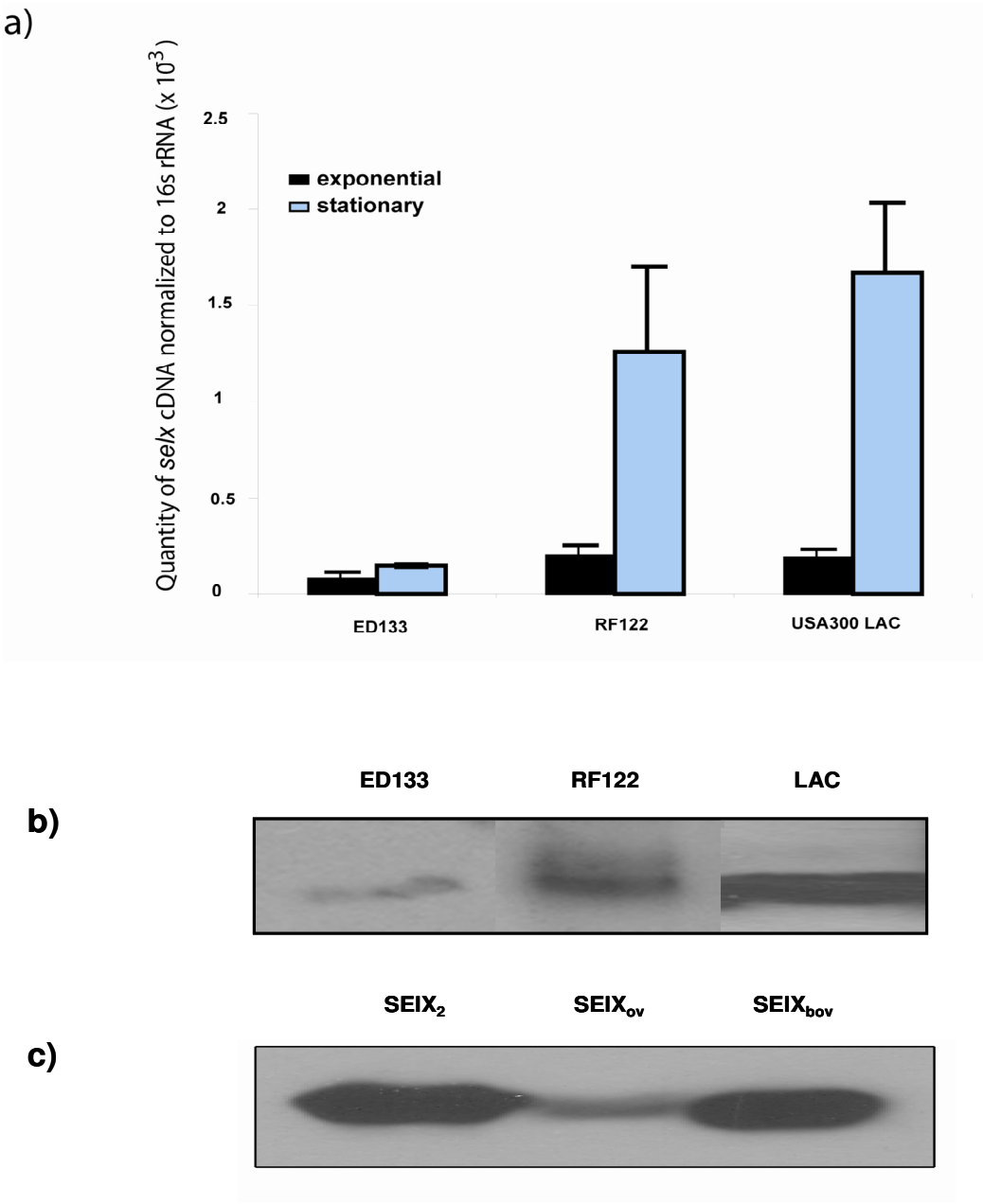

Figure 4.4. **SElX is expressed by clinical isolates *in vitro* and during infection.** **a**) Growth-phase dependent transcription of *selx*. Relative quantities of RF122 reverse-transcribed mRNA normalized to the internal control *16SrRNA*, determined by qRT-PCR. Results shown are the means of triplicate experiments and error bars indicate ± S.D. **b**) Western blot analysis of concentrated *S. aureus* supernatants of strains ED133, RF122 and LAC separated by SDS-PAGE with antibody specific for $SElX_{bov}$. c) Immunogenicity of rSElX proteins with human infective endocarditis convalescent sera. Lane 1, $rSElX_{h}$; lane 2, $rSElX_{ov}$; and lane 3, $rSElX_{bov.}$

4.3.4 SElX demonstrates Vβ-dependent mitogenicity

In addition to the recombinant bovine variant of SElX ($rSElX_{bov}$) purified in Section 3.3.6, human ($rSElX_2$) and ovine ($rSElX_{ov}$), variants of SElX were purified and used to stimulate bovine PBMCs in a thymidine incorporation assay. $rSElX_{bov}$ and $rSElX_{ov}$ were mitogenic for bovine T-cells at concentrations as low as 0.01 pg/µl, whereas $rSElX_2$ required a 10-fold higher dose of 0.1 pg/µl to induce proliferation of bovine T-cells (Figure 4.5). The bovVβ activation profile of each SElX variant was determined using the qRT-PCR assay described in Section 5.3.4 (Table 4.3). All SElX variants stimulated bovVβ subfamilies 3, 11, 17, 24 and X. In addition $SElX_{ov}$ activated bovVβ 16, $SElX_{bov}$ activated bovVβ 5 and 16, and $SElX_2$ activated bovVβ 5 and 6. Importantly, theses results demonstrate differences in mitogenic activity and distinct Vβ activation profiles for different SElX variants made by *S. aureus* strains associated with different host species.

4.3.5 SElX has a unique predicted SAg structure

SElX shares 30% and 27% amino acid identity with its closest homologs, SSL7 and TSST-1, respectively. To investigate the predicted structure of the novel SAg, SElX, we carried out structural modelling using the program Phyre (Kelley & Sternberg, 2009) with crystal structures of the most closely-related proteins in the database (TSST-1 or SSL7) as a template. The predicted structures of SElX variants indicate their potential to form a characteristic 2 domain SAg structure comprising a long central α-helical domain A with a C terminal β grasp motif, and a B domain. However the B domain of SElX is considerably smaller than the B domain for previously characterised SAgs, and lacks an N-terminal OB fold (Figure 4.6). The OB fold is involved in TSST-1 binding to the α-chain of the MHC class II complex on APC (Dinges et al., 2000, McCormick *et al.*, 2003). The functional implications of this unique predicted B domain structure are currently unknown.

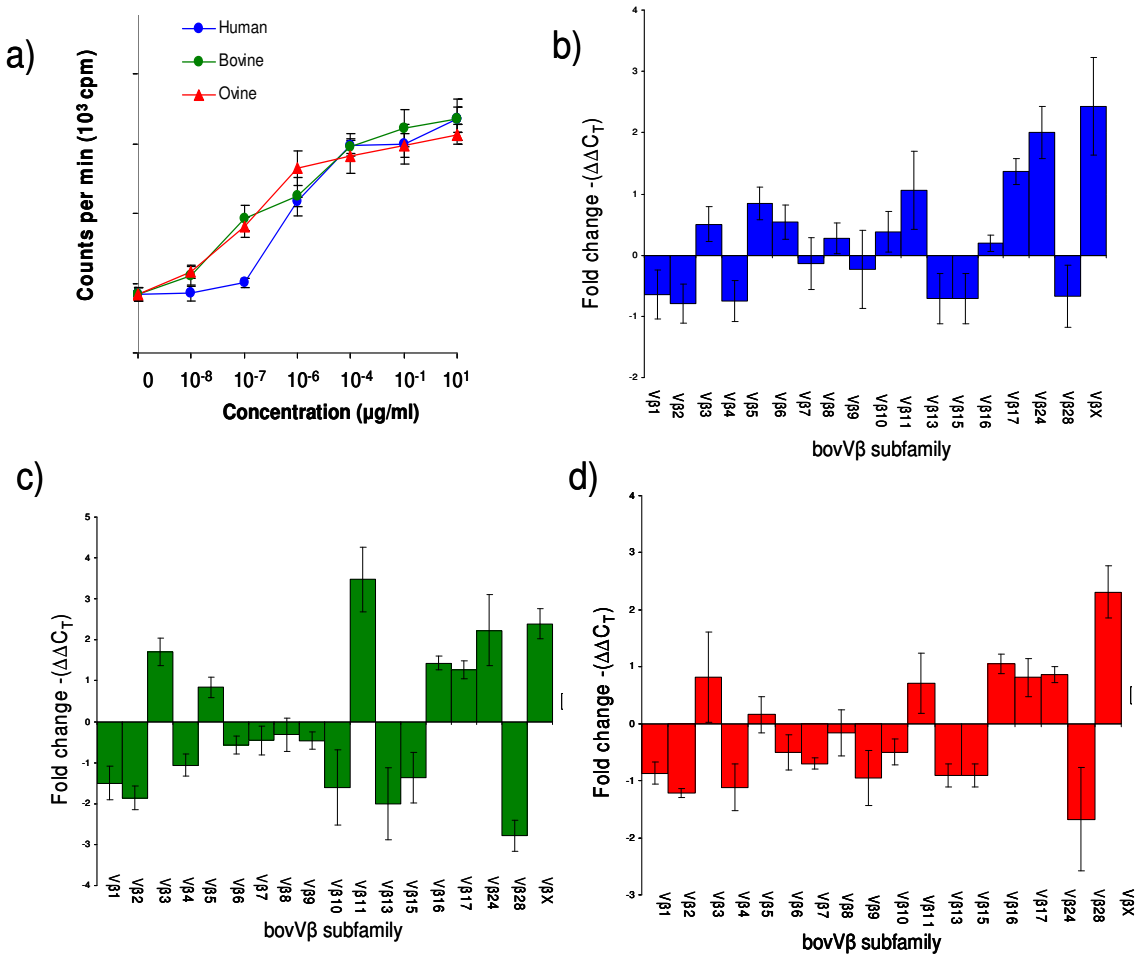

Figure: 4.5. Vβ-dependent T-cell activation of bovine PBMC cultures.

a) Proliferation of PBMCs in response to stimulation with $SEIX_{bov}$, $SEIX_2$, or $SEIX_{ov}$ after [3H]-thymidine incorporation. Relative fold-change in Vβ expression (mean ± S.E.M.) after stimulation with a final concentration of 1 µg/ml, **b)** $SEIX_2$, **c)** $SEIX_{bov}$ and **d)** $SEIX_{ov}$ for 4 d. Results shown are the means ± S.E.M. of three sets of triplicates combined from two experiments (n=6).

Table 4.3: Activation of bovVβ subfamilies in response to rSElX variants.

SElX variant	Bovine Vβ[a, b]
$SElX_2$	**3**, 5**,** 6, **11, 17, 24, X**
$SElX_{bov}$	**3**, 5, **11**, 16, **17, 24, X**
$SElX_{ov}$	**3**, **11**, 16, **17, 24**, **X**

[a] bovVβ subfamilies were named according to the nomenclature system described by Arden *et al* (Arden *et al.*, 1995).

[b] bovVβ subfamilies activated in response to the all SElX variants are highlighted in bold type.

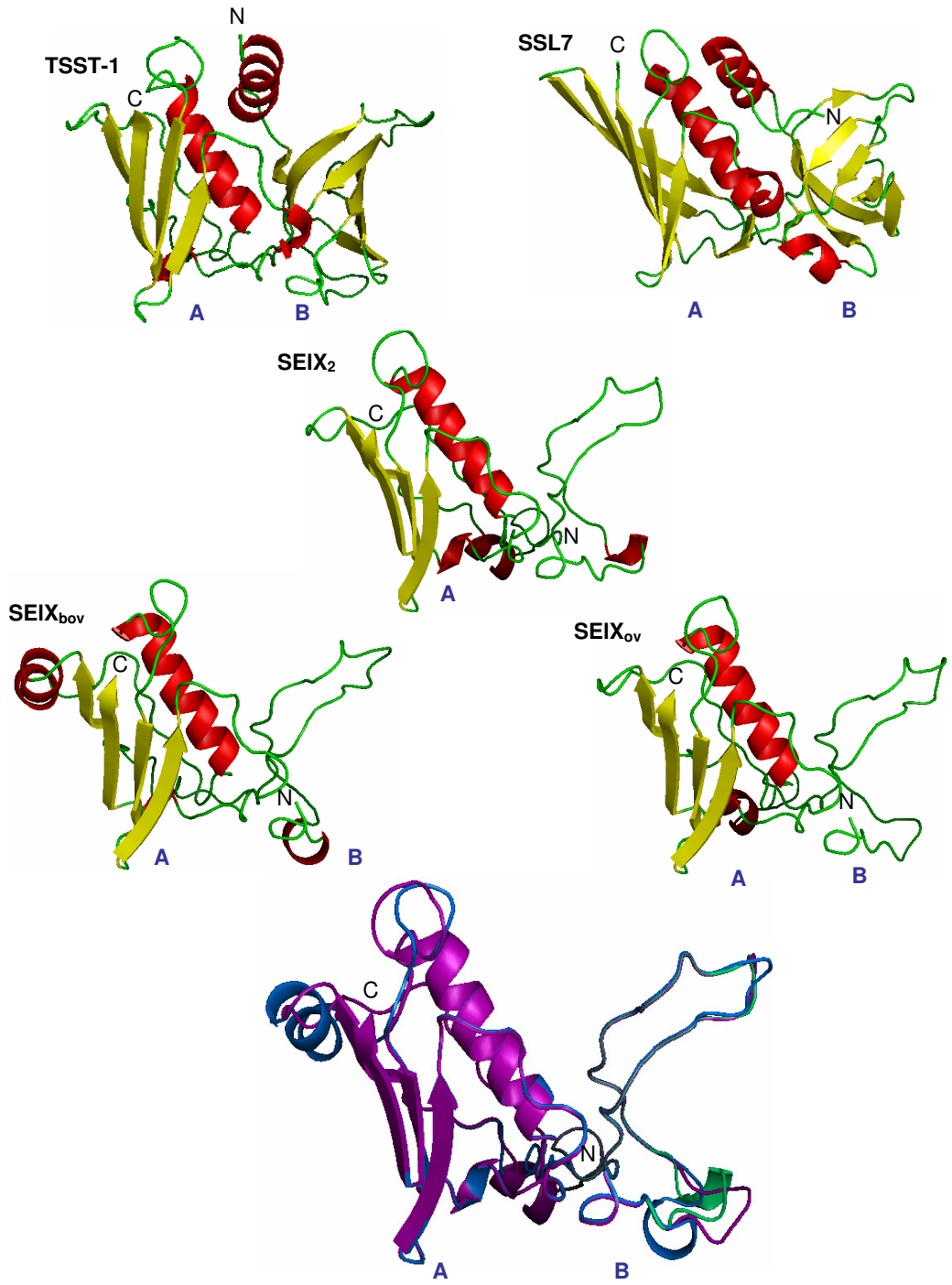

Figure 4.6: Schematic diagram of the solved structures of TSST-1, SSL7 and modelled structures of human, bovine and ovine variants of SEIX. Structural domains A and B are indicated. SEIX composite structure was obtained by superimposition of protein variants using PyMol.

4.4 Discussion

We have identified the first SAg encoded by the great majority of *S. aureus* strains. The distribution of *selx* across the full breadth of *S. aureus* diversity, its absence among other staphylococcal species and its genetic association with a gene encoding an integrase indicates an ancient horizontal acquisition event which happened prior to the most recent common ancestor of the *S. aureus* species. The gene is absent from only a single clone examined (CC30) but the associated integrase gene is retained suggesting that a deletion event has resulted in the loss of *selx* from a progenitor of the CC30 clone. Of note, previous studies have discovered that the majority of CC30 isolates contain a SaPI which encodes TSST-1, the most closely-related SAg to SElX and the only other member of the phylogenetic group IV of staphylococcal SAgs (Lindsay *et al.*, 2006, Holden *et al.*, 2004). These data imply that virtually all *S. aureus* isolates have the capacity to produce a phylogenetic group IV SAg suggesting an important role in disease pathogenesis. The gene appears to have undergone lineage-specific diversification leading to at least 14 different allelic variants identified among the strains examined (Figure 4.2).

Although SElX is the first *S. aureus* SAg identified in the core genome, the streptococcal SAg, SMEZ is found in the great majority of Group A Streptococci isolates, and exhibits extensive allelic variation leading to antigenic variation. However, SMEZ allelic variation does not influence Vβ-specificity or mitogenicity (Proft *et al.*, 2000). Whereas GAS is specific for human hosts, *S. aureus* is represented by both human, and animal clones which are most likely the result of human to animal host jumps followed by adaptive genome diversification (Lowder *et al.*, 2009). In contrast to SMEZ, SElX bovine- and ovine-specific variants displayed increased mitogenicity and a distinct Vβ profile for bovine lymphocytes in comparison to a human SElX variant suggesting that they have undergone adaptive diversification leading to enhanced activity in ruminants.

The response of human Vβ activation in response to stimulation with SElX variants was also determined in collaboration with Dr Keun Seok Seo and Prof. Greg Bohach of the University of Idaho (Table 4.4). HumVβ subfamilies 1, 6, 18 and 21 are activated by $rSElX_{bov}$ and $rSElX_2$; however $rSElX_{ov}$ activates HumVβ 1,

6 and 18 only (data not shown). These data indicate a unique pattern of Vβ gene activation for SElX in comparison to other previously characterised SAgs (Seo *et al.,* 2010, Thomas *et al*., 2009). The activation profiles of human and bovine Vβ subfamilies in response to activation with SElX are distinct. This is in part due to the activation of humVβ subfamilies 18 and 21, for which there are no bovine orthologs, and bovVβ X which has no human ortholog. However, the orthologous HumVβ 6 and bovVβ 6 are both activated by $SElX_2$. In addition the activated bovVβ subfamilies 8 and 16 are phylogenetically similar to HumVβ 6. Of note, humVβ 1 and the closely related bovVβ 5 were activated but not the orthologous bovVβ 1. However, it cannot be ruled out that other SElX variants made by human strains would have a similar functional activity for bovine lymphocytes. Previously, it was shown that the proliferation of human and bovine lymphocytes varied in response to stimulation with allelic variants of the SAg SEC. SEC_{bov} requires between 10 and 100-fold more toxin than SEC1 and SEC_{ov} to induce proliferation of human or bovine PBMCs, and different Vβ-dependent T-cell activation profiles were observed (Deringer *et al*., 1997, Marr *et al.*, 1993).

In addition to mitogenicity, in collaboration with Prof. Pat Schlievert of the University of Minnesota, Minneapolis, we have tested the pyrogenicity, lethality, and enhancement of endotoxic shock in rabbits of a representative variant $rSElX_2$ (data not shown). Pyrogenicity of $rSElX_2$ was examined in 5 rabbits after 24 h at a dose of 0.2 mg/kg. Of the 5 rabbits, 4 died within 7 d (P=0.047). In addition $rSElX_2$ stimulated enhancement of endotoxic shock. Taken together, $rSElX_2$ demonstrated functional characteristics required for classification as a SAg including mitogenicity, pyrogenicity and endotoxic shock enhancement. Additionally it would be desirable to confirm that SElX binds to MHC class II and activates T-cells without antigen processing.

Although most allelic variation in *selx* is clonal and due to point mutations, we found evidence of a role for recombination in the diversification of SElX including whole gene transfer of the *selx* allele between 2 different pathogenic clones. Although recombination rates are predicted to be low in *S. aureus* in general (Feil *et al.*, 2003), evidence for recombination, particularly among genes involved in virulence has been observed previously (Robinson *et al.*, 2005, Watanabe *et al.*,

2009). Such rapid evolution may facilitate antigenic or functional diversification of proteins which are critical for pathogenesis.

Analysis of *selx* transcription levels revealed a growth phase-dependent expression by each strain analogous to numerous other staphylococcal virulence proteins which are under the control of the *agr*. Of note, the human CA-MRSA USA300 strain LAC had very high levels of expression of SElX which correlates with the previously reported up-regulation of RNAIII and secreted virulence proteins by the USA300 epidemic clone (Li *et al.*, 2009). The USA300 epidemic clone is a major cause of mortality and morbidity in the USA and unlike most strains of hospital-acquired MRSA can cause disease in otherwise healthy individuals (DeLeo *et al.,* 2010*)*. The molecular basis for the increased virulence of USA300 strains is the subject of intensive research efforts. Several reports have highlighted the high levels of expression of secreted proteins such as PVL, α-toxin, and phenol-soluble modulins as a possible cause of the increased severity of infection associated with USA300 strains (Li *et al*., 2009, Montgomery *et al.*, 2008). Our data reveal a novel putative virulence factor made at high levels by USA300 strains which may contribute to its characteristic enhanced virulence.

In addition to demonstrating the *in vitro* expression of SElX by most *S. aureus* strains, we also identified SElX-specific antibodies in convalescent sera samples from humans, and ruminants. Due to the lack of pre-infection sera samples we cannot rule out the possibility that antibodies were induced during asymptomatic colonization of human or animal hosts rather than during disease pathogenesis. Nonetheless, our data indicate a broad role for SElX in *S. aureus* colonization or infection of multiple host species.

Structural modelling revealed SElX has the potential to form the characteristic 2 domain SAg structure. However domain B of SElX is predicted to be much smaller than that of other SAgs identified to date (Figure 4.6). Ongoing crystallography analyses should result in important insights into the molecular interaction of the uniquely structured SElX with its ligands which may facilitate the design of molecules to inhibit its function. Because of their importance in the pathogenesis of severe infection and their potential as an agent of biological warfare, therapeutic strategies which target SAgs have been the focus of considerable research efforts (Fraser & Proft, 2008). Strategies employing

Table 4.4: Activation of human and bovine Vβ subfamilies in response to rSElX variants.

SElX variant	Human Vβ[a, b]	Bovine Vβ[a]
$SElX_2$	1, 6, 18, 21	3, 5, 6, 11, 17, 24, X
$SElX_{bov}$	1, 6, 18, 21	3, 5, 11, 16, 17, 24, X
$SElX_{ov}$	1, 6, 18	3, 11, 16, 17, 24, X

[a] Vβ subfamilies were named according to the nomenclature system described by Arden *et al* (Arden et al., 1995).

[b] HumVβ activation was determined by Keun Seok Seo, University of Idaho.

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vaccination, passive immunization, peptide antagonists and receptor mimics have been developed with limited success (Fraser & Proft, 2008). Importantly, the diversity of SAgs made by different strains and their distinct Vβ-dependent binding activities has complicated efforts to design broadly effective therapeutics.

In summary, we have discovered a novel SAg made by the great majority of *S. aureus* clinical isolates which is involved in immune modulation during human and animal infections. The discovery may have important implications for our understanding of *S. aureus* disease pathogenesis

Chapter 5
Comprehensive analysis of bovine T-cell reactivity with staphylococcal superantigens

5.1 Introduction

Vβ specific activation of human T-cells in response to staphylococcal SAgs has been well characterised (Seo *et al*., 2010, Thomas *et al*., 2009, Ono *et al*., 2008), but the bovine T-cell response to SAgs is poorly understood. Previously, SEC and TSST-1 have been shown to induce Vβ specific proliferation of bovine T-cells (Deringer *et al*., 1997, Deringer *et al.*, 1996, Fitzgerald *et al*., 2001a). However these studies were limited by the number of TRBV gene sequences available, and only 5 subfamilies VB1, 2, 4, 13, and 28 were included. The recent bovine genome sequencing project and cDNA analyses led to the identification of the full complement of bovine Vβ subfamilies and almost the entire repertoire of bovine TRBV genes (Connelley *et al*., 2009, Elsik *et al*., 2009). This has facilitated the development of qRT-PCR assay to study the bovVβ response to stimulation with staphylococcal SAgs.

The aim of this study was to investigate the effect of SAgs on the bovine T-cell response by:

- Examining mitogenic potential of RF122-encoded SAgs for bovine T-cells.

- Determining the bovVβ specific T-cell activation profile of individual SAgs.

5.2 Materials and Methods

5.2.1 Bacterial strains and plasmids

Bacterial strains and plasmids used are shown in Table 5.1. Media were supplemented where appropriate with chloramphenicol at 10 μg/ml (*S. aureus*) or 200 μg/ml (*Escherichia coli*), 50 μg/ml ampicillin, and 150 μg/ml X-gal.

5.2.2 Cloning of SAg genes into expression plasmid pALC2073

Primers were designed to amplify RF122-encoded SAg genes for cloning into the expression plasmid pALC2073. Each 5´ oligo was designed to prime upstream of the predicted ribosome binding site (RBS) with a *Kpn*I site incorporated to facilitate cloning. The 3´ primer was designed to include the stop codon of the gene with a *Sac*I site incorporated. A schematic diagram of the cloning procedure is presented in Figure 5.1. PCR reactions were carried out as described in General Methods with 100 nmol forward and reverse primers, as listed in Table 5.2, using 1 U Vent polymerase (New England Biolabs, Herts, UK). PCR products were cloned into the pSC-B blunt cloning vector as described in the manufacturer's instructions (Stratagene, Agilent, UK). SAg gene inserts were released from the pSC-B plasmids by digestion with *Sac*I and *Kpn*I for 3 h at 37 °C, purified by gel extraction, ligated with digested pALC2073 plasmid DNA using T4 DNA ligase, and transformed into *E.coli* DH5α. The resulting pALC2073::SAg plasmids were isolated from DH5α and transformed by electroporation into an intermediate electrocompetent strain of *S. aureus,* RN4220. Subsequently the plasmids were re-isolated and transformed into the SAg and α toxin-deficient strain RF122-8α, the construction of which is described in Section 6.3.1.

5.2.3 Expression of SAg proteins in RF122-8α

RF122-8-α strains containing each of the pALC2073::SAg constructs were induced with 50 ng/ml, a sub-inhibitory concentration of tetracycline (Sigma-Aldrich, Dorset, UK) when cultures reached mid-exponential phase, and grown for a further

Table 5.1: Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source/Reference
S. aureus		
RN4220	Restriction/modification$^-$	(Kreiswirth *et al.*, 1983)
RF122	Bovine mastitis	(Fitzgerald *et al.*, 2000)
RF122t-α	Δ*hla*	Section 6.3.4
RF122-7	*tst*::Tc^{r}Δ*sec*Δ*sell*Δ*egc*Δ*selx* Δ*sely*	Section 6.3.1
RF122-8	*tst*::Tc^{r}Δ*sec*Δ*sell*Δ*egc*Δ*selx* Δ*sely*Δ*selz*	Section 6.3.1
RF122-8α	*tst*::Tc^{r}Δ*sec*Δ*sell*Δ*egc*Δ*selx* Δ*sely*Δ*selz*Δ*hla*	Section 6.3.4
RF122-8α-C	RF122-8α containing pALC2073::SEC_{bov}	This study
RF122-8α-L	RF122-8α containing pALC2073::$SElL_{bov}$	This study
RF122-8α-T	RF122-8α containing pALC2073::TSST-1	This study
RF122-8α-G	RF122-8α containing pALC2073::SEG_{bov}	This study
RF122-8α-I	RF122-8α containing pALC2073::SEI_{bov}	This study
RF122-8α-U	RF122-8α containing pALC2073::$SElU_{bov}$	This study
RF122-8α-N	RF122-8α containing pALC2073::$SElN_{bov}$	This study
RF122-8α-O	RF122-8α containing pALC2073::$SElO_{bov}$	This study
RF122-8α-X	RF122-8α containing pALC2073::$SElX_{bov}$	This study

Strain or plasmid	Description	Source/Reference
RF122-8α-Y	RF122-8α containing pALC2073::SElY$_{bov}$	This study
RF122-8α-Z	RF122-8α containing pALC2073::SElZ$_{bov}$	This study
E. coli		
DH5α	Cloning strain	Invitrogen
Strataclone SoloPack™	*lacZΔM15* mutation, *endA*, *recA* deficient	Stratagene
Plasmids		
pALC2073	Expression plasmid; pSK236 containing the *tetR* gene and the *xyl/tetO* promoter	(Bateman *et al.*, 2001)
pALC2073::SEC$_{bov}$	*secbov* cloned into pALC2073	This study
pALC2073::SElL$_{bov}$	*sellbov* cloned into pALC2073	This study
pALC2073::TSST-1$_{bov}$	*tstbov* cloned into pALC2073	This study
pALC2073::SEG$_{bov}$	*segbov* cloned into pALC2073	This study
pALC2073::SEI$_{bov}$	*seibov* cloned into pALC2073	This study
pALC2073::SElO$_{bov}$	*selobov* cloned into pALC2073	This study
pALC2073::SElN$_{bov}$	*selnbov* cloned into pALC2073	This study
pALC2073::SElU$_{bov}$	*selubov* cloned into pALC2073	This study

Strain or plasmid	Description	Source/Reference
pALC2073::SElY$_{bov}$	*selybov* cloned into pALC2073	This study
pALC2073::SElZ$_{bov}$	*selzbov* cloned into pALC2073	This study
pALC2073::SElX$_{bov}$	*selxbov* cloned into pALC2073	This study
pSC-B	Contains *lacZ*' α-complementation cassette	Stratagene

Table 5.2: Primers used to clone SAg genes encoded by RF122 into the *Sac*I and *Kpn*I sites of pALC2073.

Primer [a]	Sequence (5´ - 3´) [b]
secbov-F	TCTAGGTACCTCTTGGGAATGTTGG
secbov-R	TATCGAGCTCGGATTAACATTATCC
sellbov-F	TTATGGTACCTATAATGAAAAGGAAGTGC
sellbov-F	AATCTGAGCTCTAATAATTGGAATCATC
tstbov-F	AAACGGTACCACATTTAAATGAAGG
tstbov-R	AAAAGAGCTCAATTAATTAATTTCTGC
segbov-F	CATTGGTACCTAGACTGAATAAGTTAGAGG
segbov-R	ATTTGAGCTCTTCAGTAAATTTTATATATTC
seibov-F	CAATGGTACCTTAGAAAAGGAAATGC
seibov-R	TTTAGAGCTCAAACTAATTATCATTAG
selobov-F	TTTTGGGTACCAGGGATATTATAA
selobov-R	TTGTGAGCTCATATGCATCAACTT
selnbov-F	TTGAGGTACCGTATATTATAAA
selnbov-R	GATTGAGCTCATAATCATCAATCACTT
selubov-F	TACCTGGTACCCATCAAATTTATAAA
selubov-F	TAATGAGCTCTCCCTCAATTA
selxbov-F	TAATGGTACCAAATAAAGCTTTACATC
selxbov-R	AATTGAGCTCAATTTCAAACTTGTTC
selybov-F	CAATGGTACCTGAAGGTCAACTAA
selybov-R	TAAGGAGCTCTATGTCTACTTTTTAGTT
selzbov-F	TTTGGTACCTTTAATCTTAAATGAGA
selzbov-R	GGAACGAGCTCTTTCATATAAATATCT
pALCMCS-F	ATACCGCACAGATGCGTAAGG
pALCMCS-R	CGATGACTTAGTAAAGCACATCTAA

[a] F, forward primer, R, reverse primer.

[b] Restriction sites incorporated are underlined.

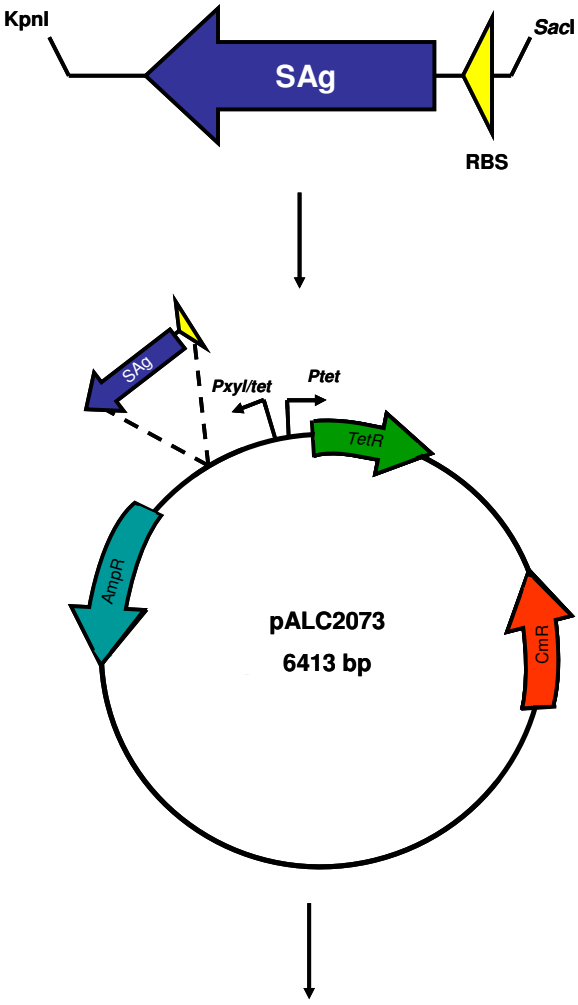


Figure 5.1: Schematic diagram of the procedure used to clone SAg genes encoded by RF122 into expression plasmid pALC2073.

4 h (Bateman B.T *et al*, 2001). Cells were centrifuged at 4000 x g and supernatant fractions containing secreted proteins were removed and concentrated with Amicon Ultra-15 Centrifugal Filter units with a 10 kDa MWCO as described in the manufacturers instructions (Millipore, Watford, UK). RF122-8α containing pALC2073 was used as a negative control for induction. Concentrated secreted proteins were separated on 10% SDS-PAGE gels, stained overnight at room temperature with Coomassie Blue (Severn Biotech), or transferred to Nitrocellulose membranes (Amersham Hybond™ ECL™, GE Healthcare, Slough, UK) for Western blot analysis. The membrane was incubated with primary antibody for 1 h with 1:2500 dilution of anti-SEC (Santa Cruz Biotechnology, Heidelberg, Germany), or 2 h with a 1:2000 dilution of rat antisera specific for rTSST-1, rSE*l*L, or rSE*l*X_{bov} (provided by K.S Seo, University of Idaho). Membrane was incubated with secondary antibody for 1 h at dilutions; 1:2500 (Rabbit anti-mouse IgG, Zymed, Invitrogen, UK), or 1:1500 Goat polyclonal antibody to rat IgG/HRP, (Abcam, Cambridge, UK), and visualised by ECL.

5.2.4 T-cell proliferation assays

Blood was obtained from Holstein–Friesian cattle, animals, 851, 1683 and 1693 aged 18 to 36 months via jugular vein puncture, and PBMC were isolated by density gradient centrifugation as described in General Methods. PBMC adjusted to a concentration of 1×10^6 cells/ml in complete cell culture medium, were stimulated in triplicate at least with 10-fold dilutions (0.01 ng/ml to 100 ng/ml) of concentrated *S. aureus* supernatant fractions. Culture media and 50 µg/ml Concanavalin A were used as negative and positive controls respectively. Proliferation of Bovine PBMC was assessed by [^{3}H]-thymidine incorporation assay or FACS analysis of CFSE stained cells as described in General Methods.

5.2.5 Analysis of Vβ T-cell activation

Total RNA was extracted from bovine PBMC (4×10^6 cells) using Tri-reagent (Sigma–Aldrich, Dorset, UK) as described in the suppliers instructions. First-strand cDNA was generated from 0.5 µg of RNA using Power SYBR® Green RNA-to-CT 2-Step Kit (Applied Biosystems, Warrington, UK). The reverse transcription

reaction was performed in a 20 µl volume according to the manufacturer's specifications. Bovine Vβ subfamily specific qRT-PCR primers were designed with Primer3 (Rozen, 2000), based on an alignment of bovine Vβ sequences. cDNA sequences for bovine TRBV genes were derived from the bovine genome and cDNA analyses described by Connelley *et al* (Connelley *et al*., 2009). To quantify cDNA generated by reverse transcription from target RNA, qRT-PCR reactions were carried out in 25 µl reactions containing 100 ng cDNA, 100 nM primers (Table 5.3), and SYBR Green I dye master mix (AB) as per the manufacturers instuctions, using a Stratagene Mx3000P light cycler. The thermal conditions were: 1 cycle at 50 °C for 10 min, 1 cycle at 95 °C for 10 min, 15 s at 95 °C and 1 min at 60 °C for 40 cycles. RNA samples were processed in triplicate with NTC and no RT controls.

qRT-PCR data were analyzed using MxPro software version 4.1 (Strategene, Agilent Technologies UK Ltd. Cheshire). The threshold cycle (C_T) was determined and normalized to internal controls, β-actin and Constant β (Cβ), by calculating ΔC_T [$C_{T\ target}$ - $C_{T\ \beta\text{-actin}}$- $C_{T\ (\beta\text{-actin}\ -\ C\beta)}$). Specificity of the qRT-PCR products was determined by melt curve analysis. Normalized ΔC_T data were then compared by calculating $\Delta\Delta C_{T\ =}$ -(ΔC_T stimulated - ΔC_T of unstimulated). Values >0 reveal expansion of particular subset, whereas values equal to or < 0 reveal non expansion of particular subset by stimulation with concentrated RF122-8α supernatants containing expressed individual SAgs.

Table 5.3: Primers used to identify bovVβ activation

Vβ subfamily	Forward Sequence (5'-3')	Reverse Sequence (3'-5')
Vβ 1 (9)	CTGATCAAATCAAGAAAASCAGCAAG	CTGAGTCTGYCAGCTCCAAG
Vβ 2 (20)	CGCTCTCGTCTCTCAGCAG	TAACCTTGTTCGTATGTGGCATC
Vβ 3 (28)	GCTGCTCCATTTCTCAATCGA	GGCACATCCCCTTCCTCAA
Vβ 4 (29)	CTCCTGGGACTGGGTTCTG	AGCCCTGATTGGCAGTAGC
Vβ 5 (5)	CCTATGTCTGGACACAGCAGTG	TCGAAAAGTTTCCTTTAGCTCTTTG
Vβ 6 (7)	GGGAGGGGCCAGACTGTAA	TTTAGGCATYCCTGATTTGTC
Vβ 7 (4)	CTCAGATACCAAAATACCTAGTCATG	GAACACTCTCATTTCCAGTGAGTTTC
Vβ 8 (12)	GTCACTCTGAGTTGTGAGCYGA	CCGACTCATCAATAGGAGCTTG
Vβ 9 (3)	CCTCACACAGATGGGAACTAAGAC	GCACCGTTTCATTTCCAACG
Vβ 10 (21)	AGCAAAGATGGATTGTGTCCC	GGTGGAGTTGATTTCCAGGCT
Vβ 11 (25)	TCAAACTATGGGCCTTGACAAC	GCTCTCCTTTCTCCGTGGTATTAAC
Vβ 13 (6)	CCAKGTCGTGAGGASAGGA	CTGAGTAATGGATYAGCCTCAG
Vβ 15 (24)	CACAGGAAAGAGCACTGTACTGG	CATCTCCTTTGTTAATATCATC
Vβ 16 (14)	GACCCTATTTCTGGACATGAATCT	CCTTTGGGCATCCCTGAGT
Vβ 17 (19)	GTGACCCTGGARTGTRAACAG	TCWCGAGAGRCRCTGTAGC
Vβ 24 (15)	TACCGGGCTGGGAAAACC	TGTTTTTTTCCTTGGTGATTTGTTTA
Vβ 28 (26)	CGCAGGATCTTGGAGACTGA	TCTTAAAGGAGTCAGGGCCAGTT
Vβ X (X)	CTACAGGTGCTGGCCAGTCTG	GGTTTATGACTTCCTTATCCCCGG
Constant β	CCTGGTGGGTGAACAGGAAG	CCTCCCACTGGTCCTGGTC
β-actin	GGCCGAGCGGAAATCG	GCCATCTCCTGCTCGAAGTC

[a] Vβ subfamily nomenclature followed the classification of Arden *et al* (Arden *et al*., 1995), with the IMGT classification shown in brackets (Folch & Lefranc, 2000).

5.3 Results

5.3.1 Expression of individual RF122-encoded SAgs in the SAg-deficient strain RF122-8α

To investigate the effect of individual RF122-encoded SAgs on bovine T-cells *in vitro*, SAg genes were cloned into the expression plasmid pALC2073, which has an inducible promoter, to allow controlled expression in a SAg-deficient strain, RF122-8α, the construction of which is described in Chapter 6. RF122 SAg genes; *secbov, sellbov, tstbov, segbov, seibov, selobov, selnbov, selubov, selxbov, selybov,* and *selzbov* encoded by RF122 were cloned into the *Sac*I and *Kpn*I sites of pALC2073 resulting in constructs; pALC2073::SEC_{bov}, pALC2073::$SElL_{bov,}$ pALC2073::$TSST\text{-}1_{bov,}$ pALC2073::SEG_{bov}, pALC2073::SEI_{bov}, pALC2073::$SElO_{bov}$, pALC2073::$SElN_{bov,}$ pALC2073::$SElU_{bov}$, pALC2073::$SElX_{bov}$, pALC2073::$SElY_{bov}$, and pALC2073::$SElZ_{bov}$, respectively (Table 5.1). Constructs were verified by restriction digestion with *Sac*I and *Kpn*I, which revealed inserts of the expected size (Figure 5.2). DNA sequencing reactions were also carried out with MCS primers (Table 5.2).

pALC2073::SAg constructs and pALC2073 were transformed into RF122-8α, and cultures were induced with a sub-inhibitory concentration of tetracycline. To determine whether SAg proteins are expressed under the control of the pALC2073 plasmid promoter, pALC2073::SAg supernatant fractions were compared with supernatant from a culture of pALC2073 only (Figure 5.3). Proteins of the predicted molecular weight were expressed in supernatants of induced RF122-8α cultures containing, pALC2073::SEC_{bov} (27.6 kDa) pALC2073::$SElL_{bov}$ (24.7 kDa), pALC2073::$TSST\text{-}1_{bov}$ (22 kDa), pALC2073::$SElU_{bov}$ (27.2 kDa), $SElY_{bov}$ (27.1 kDa) and $SElZ_{bov}$ (22.5 kDa), as indicated in Figure 5.3.

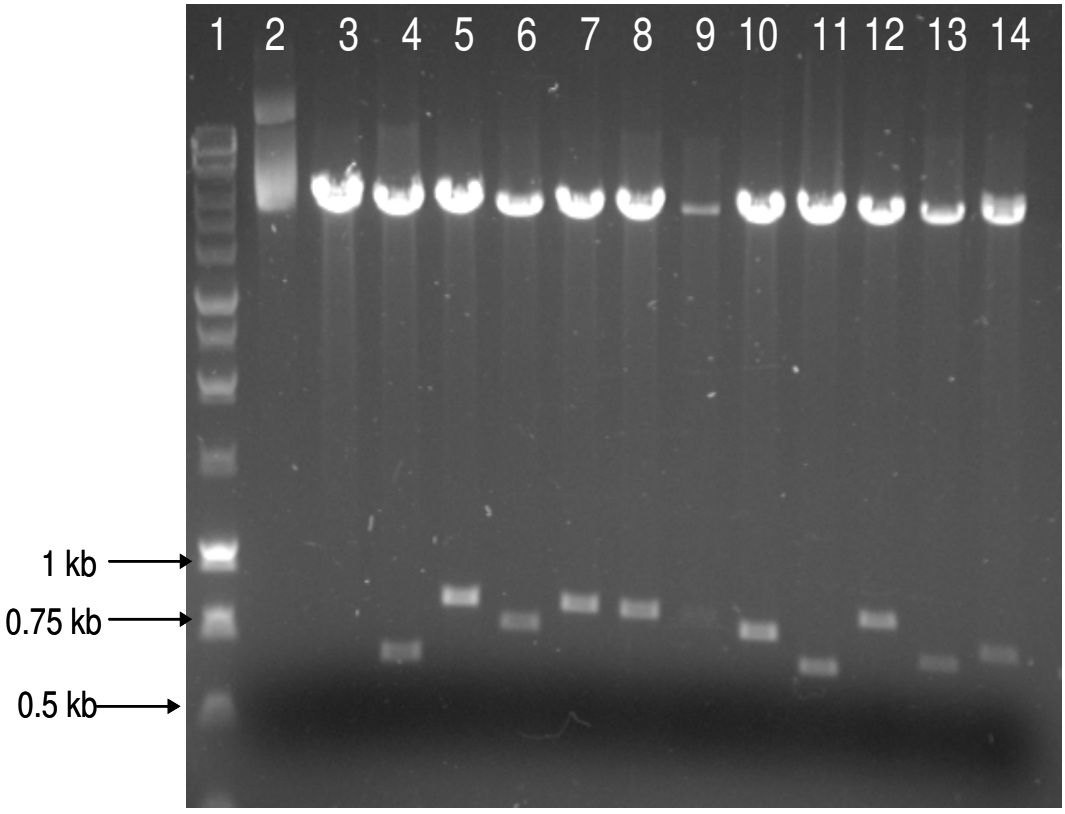


Figure 5.2: Verification of pALC2073 constructs containing RF122-encoded SAg genes by digestion with *Sac*I and *Kpn*I. Lane 1, 1 kb+ ladder; lane 2, undigested pALC2073; lane 3, digested pALC2073; lane 4, pALC2073::TSST-1_{bov}; lane 5, pALC2073::SEC_{bov}; lane 6, pALC2073::$SElL_{bov}$; lane 7, pALC2073::$SElU_{bov}$; lane 8, pALC2073::$SElN_{bov}$; lane 9, pALC2073::$SElO_{bov}$, lane 10, pALC2073::SEI_{bov}; lane 11, pALC2073::SEG_{bov}; lane 12, pALC2073::$SElY_{bov}$; lane 13, pALC2073::$SElX_{bov}$; and lane 14, pALC2073::$SElZ_{bov}$.

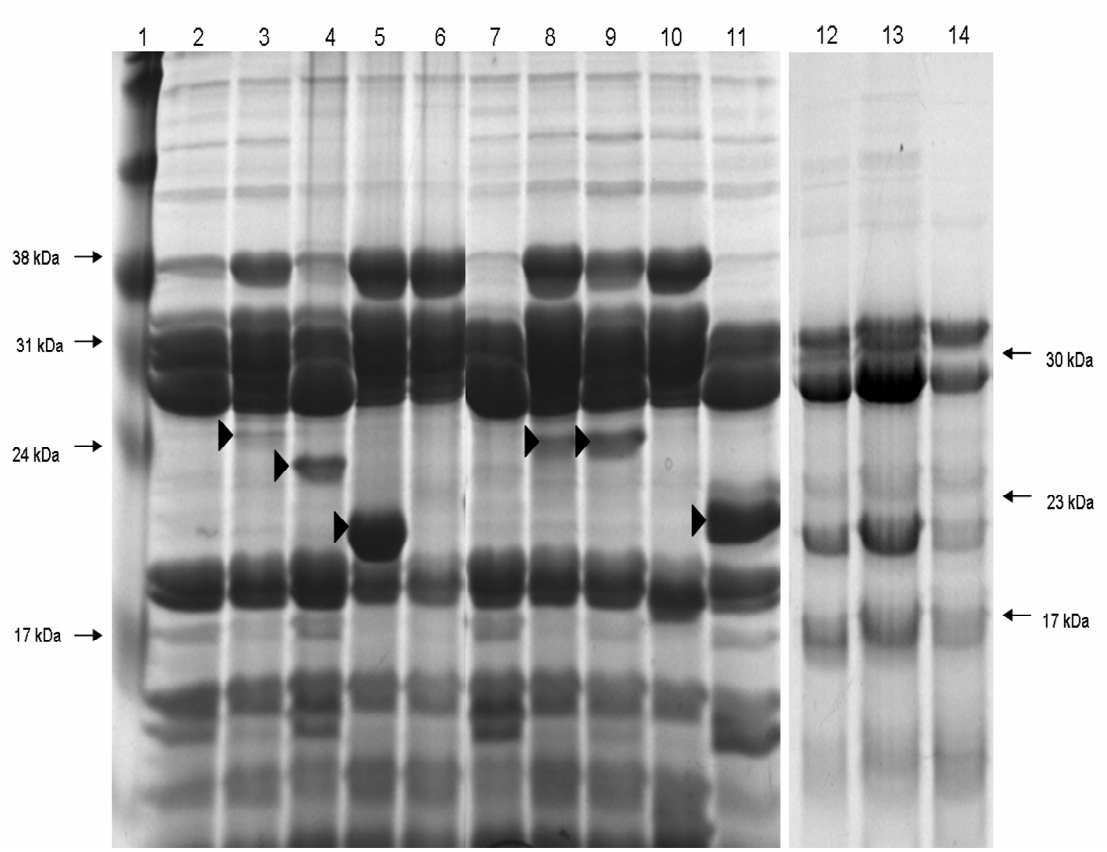


Figure 5.3: Expression of SAg genes in RF122-8α. RF122-8α supernatants expressing individual RF122-encoded SAgs, concentrated approx. 100-fold and resolved on SDS-PAGE gels and stained with Coomassie. Lane 1, Full range Rainbow ladder; lane 2, RF122-8α containing pALC2073; lane 3, RF122-8α-C, lane 4, RF122-8α-L; lane 5 RF122-8α-T; lane 6, RF122-8α-G; lane 7, RF122-8α-N; lane 8, RF122-8α-U; lane 9, RF122-8α-Y; lane 10, RF122-8α-I; lane 11, RF122-8α-Z; lane 13, RF122-8α; lane 14, RF122-8α-X; lane 15, RF122-8α-O. Black arrows indicate where SAg proteins of the predicted molecular weight were expressed.

However it was not possible to visually detect expression of $SEG_{bov,}$ $SElN_{bov}$, $SEI_{bov,}$ $SElO_{bov}$ or $SElX_{bov}$. $SElX_{bov}$ which is 19.5 kDa in size was not detectable on the Coomassie stained gel, but was detected by western blot analysis with rat anti-sera specific for $rSElX_{bov}$ (Figure 5.4). In addition western blot analysis was used to confirm expression of SaPIbov SAgs; SEC_{bov}, $SElL_{bov}$ and $TSST\text{-}1_{bov}$ (Figure 5.4). Primary antibodies specific for $SEG_{bov,}$ $SElN_{bov}$, $SEI_{bov,}$ and $SElO_{bov}$ were not available to confirm expression.

5.3.2 RF122-encoded SAgs are mitogenic for bovine T-cells

To examine if RF122-encoded SAgs are mitogenic for bovine T-cells, culture supernatants of RF122-8α containing pALC2073::SAg constructs were used to stimulate bovine PBMC and proliferation was measured using a thymidine incorporation assay (Figure 5.5). Previously, superantigenic activity of SEC_{bov}, $TSST\text{-}1_{bov}$ had been described (Fitzgerald *et al*., 2001a, Deringer *et al*., 1997, Deringer *et al*., 1996). Here, we have shown that in addition to SEC_{bov} and $TSST\text{-}1_{bov,}$ bovine variants of characterised SAgs, $SElL_{bov}$ SEI_{bov}, $SElN_{bov,}$ and novel SAgs, $SElX_{bov}$ and $SElY_{bov}$ are mitogenic for bovine T-cells. RF122 and RF122-8α supernatants containing SEC_{bov}, $TSST\text{-}1_{bov,}$ $SElL_{bov}$ $SElN_{bov,}$ $SElX_{bov}$ and $SElY_{bov}$ induced proliferation at concentrations ranging from 10 pg/µl to 0.01µg/ml (Figure 5.5). SEI_{bov} induced proliferation at concentrations ranging from 100 pg/µl to 0.01µg/ml. However proliferation was not induced by 10 pg/µl to 0.01µg/ml RF122-8α supernatants containing $SElO_{bov}$, SEG_{bov} , $SElU_{bov}$ or $SElZ_{bov.}$ FACS analysis of CFSE stained PBMC stimulated with 10 ng/ml culture supernatants was also carried out, where proliferation was assessed by measuring loss of fluorescence in dividing cells. SEC_{bov}, $TSST\text{-}1_{bov,}$ $SElL_{bov,}$ SEI_{bov}, $SElN_{bov,}$ $SElX_{bov}$ and $SElY_{bov}$ induced proliferation of bovine T-cells, which confirmed the results of the thymidine incorporation assay (Figure 5.6).

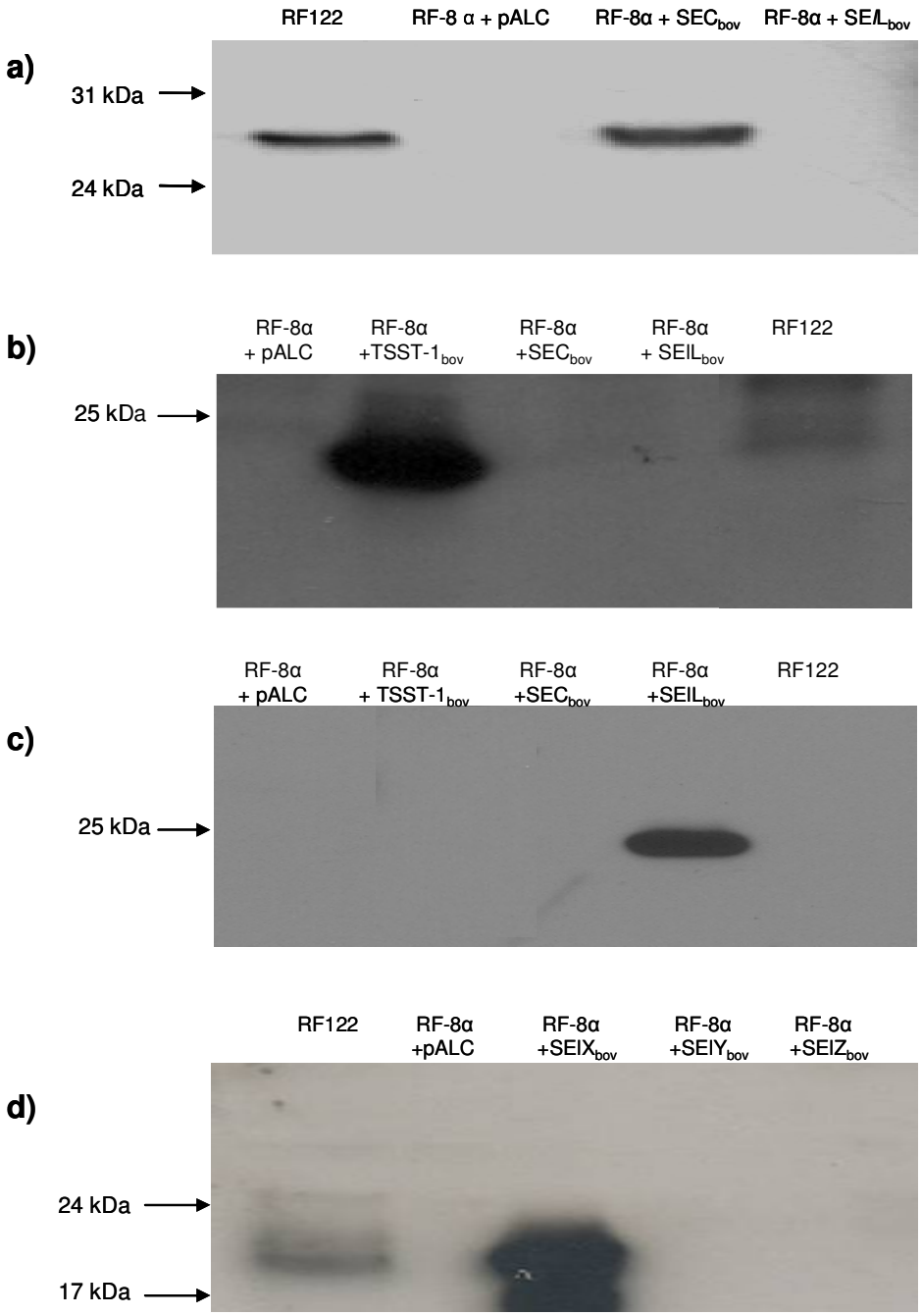


Figure 5.4: Western blot analysis of SEC$_{bov}$, TSST-1$_{bov}$, SEIL$_{bov}$ and SEIX$_{bov}$ expression in RF122-8α. Western blot analysis of concentrated RF122-8α supernatants containing SAgs separated by SDS-PAGE, with antibody specific for **a)** SEC$_{bov}$, **b)** TSST, **c)** SEIL or **d)** SEIX$_{bov}$.

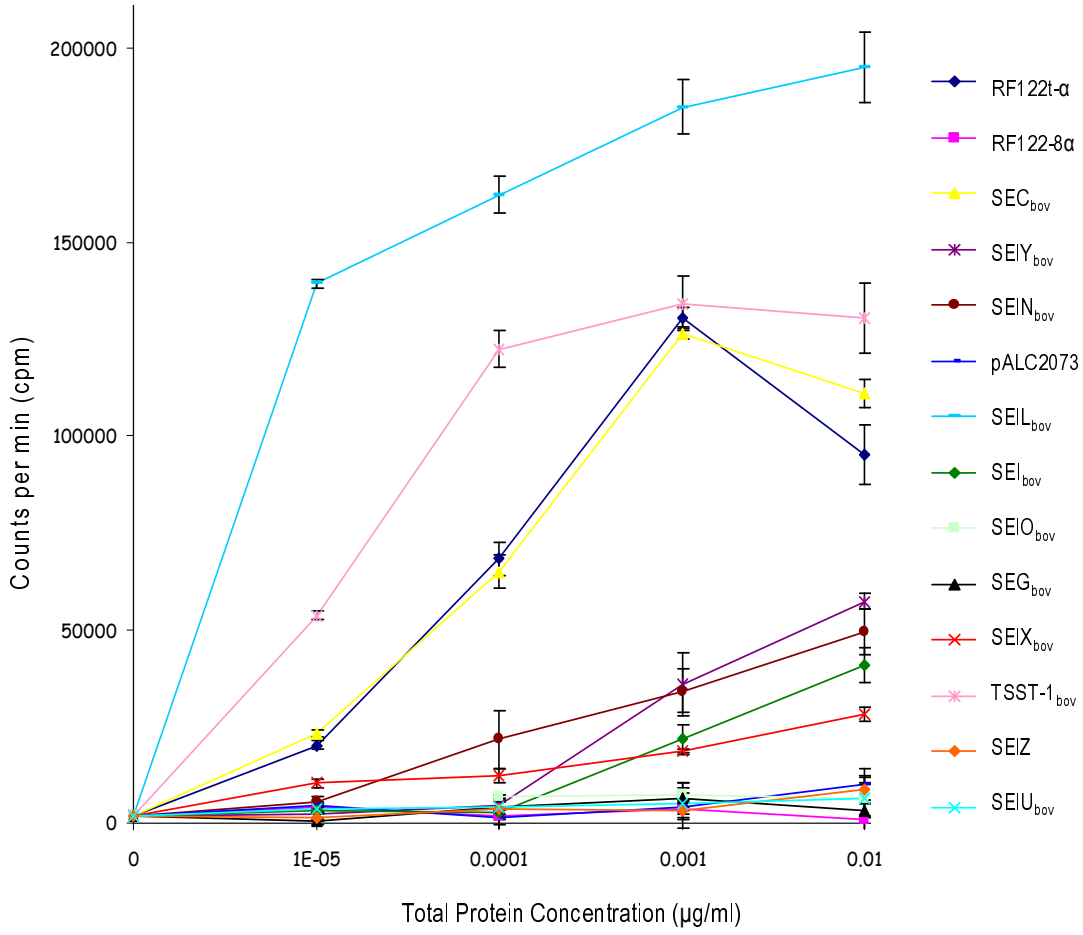


Figure 5.5: Proliferation of bovine T-cell populations in response to stimulation with RF122-encoded SAgs. PBMC proliferation after 4 d exposure to RF122-8α supernatants containing SAgs, as indicated by the incorporation of [^{3}H] thymidine. Results shown are the means of triplicate measurements from 2 animals ± SD.

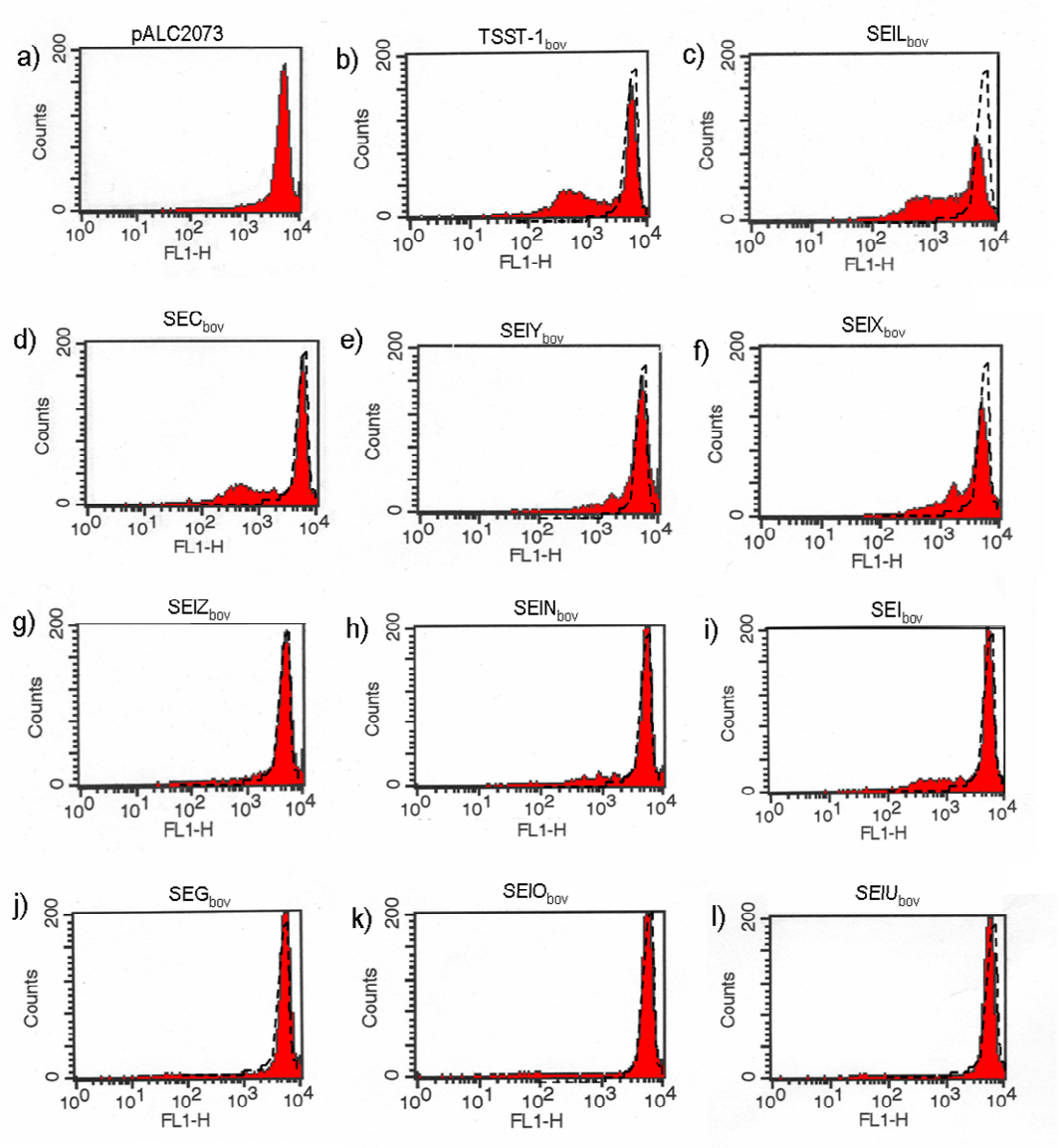


Figure 5.6: T-cell proliferation in response to stimulation with RF122-encoded SAgs assessed by CFSE staining. Bovine PBMC were labelled with CFSE prior to culture and stimulated for 4 d with 10 ng/ml culture supernatants of; **a)** RF122-8α containing; pALC2073, **b)** RF122-8α-T, **c)** RF122-8α-L, **d)** RF122-8α-C, **e)** RF122-8α-Y, **f)** RF122-8α-X, **g)** RF122-8α-Z, **h)** RF122-8α-N, **i)** RF122-8α-I, **j)** RF122-8α-G, **k)** RF122-8α-O and **l)** RF122-8α-U. Broken lines indicate the data obtained for stimulation with pALC2073 only.

RF122-8α supernatant was toxic for bovine PBMC at concentrations over 0.1 μg/ml, meaning that proliferation could not be measured. Consequently, it is possible that $SElO_{bov}$, $SEG_{bov,}$ $SElU_{bov}$ and $SElZ_{bov}$ require a higher concentration than was tested to induce proliferation. Our data indicate that $SElU_{bov}$ and $SElZ_{bov}$ are not mitogenic at the concentrations examined, but expression of $SElO_{bov}$ and SEG_{bov} in RF122-8α has not been confirmed and it is therefore not possible to conclude a lack of mitogenic activity. Of note, SElU is mitogenic for human T-cells (Thomas *et al*., 2006, Thomas *et al*., 2009), but $SElU_{bov}$, which is encoded by an allelic variant is not mitogenic for bovine T-cells. In contrast to the lack of T-cell proliferation observed during stimulation with RF122-8α supernatant containing $SElZ_{bov}$, data obtained for isogenic mutant RF122-8 deficient in $SElZ_{bov}$ expression compared with RF122-7 (construction of which is described in Section 6.3.1), indicates that $SElZ_{bov}$ is mitogenic (Figure 5.7). This discrepancy may be explained by the fact that culture supernatants were obtained from different phases of growth. RF122-7 supernatant was obtained from post-exponential phase whereas RF122-8α-Z supernatant was harvested from stationary phase. This implies that $SElZ_{bov}$ superantigenic activity may require the presence of another factor expressed in stationary phase.

5.3.3 RF122-encoded SAgs activate unique bovVβ profiles

Previous studies of the bovine Vβ-dependent expansion capacity of staphylococcal SAgs have been restricted by the number of identified bovine Vβ subfamilies (Fitzgerald *et al*., 2001a, Deringer *et al*., 1997). The recent bovine genome sequencing project has facilitated identification of almost the entire TRBV repertoire (Elsik *et al*., 2009, Connelley *et al*., 2009). Connelley *et al* recently established an RT-PCR assay to identify clonal expansion within the full complement of bovVβ subfamilies (Connelley *et al.*, 2008). However this assay is not quantitative, so to quantify the expansion of bovine Vβ subfamilies in response to stimulation with RF122-encoded SAgs, we have developed a novel qRT-PCR assay which includes the complement of bovine Vβ subfamilies. We have used the Vβ nomenclature scheme proposed by Arden *et al*, to allow comparison with previous studies which have determined humVβ activation in response to SAgs (Thomas *et al*., 2009, Seo *et al*., 2010, Arden *et al*., 1995). 18 Vβ

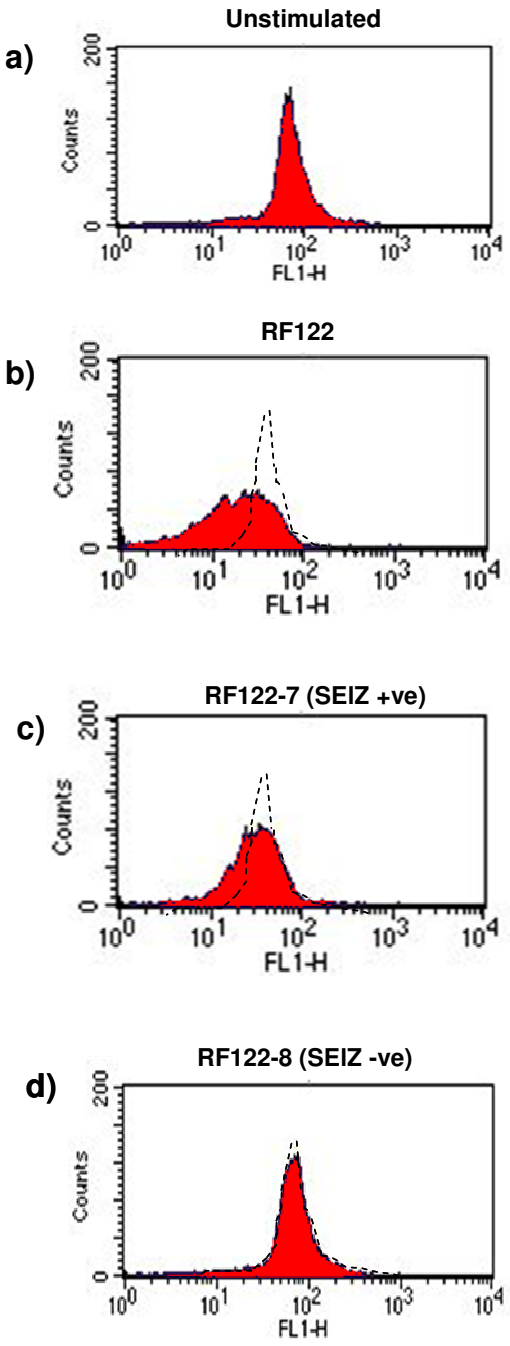
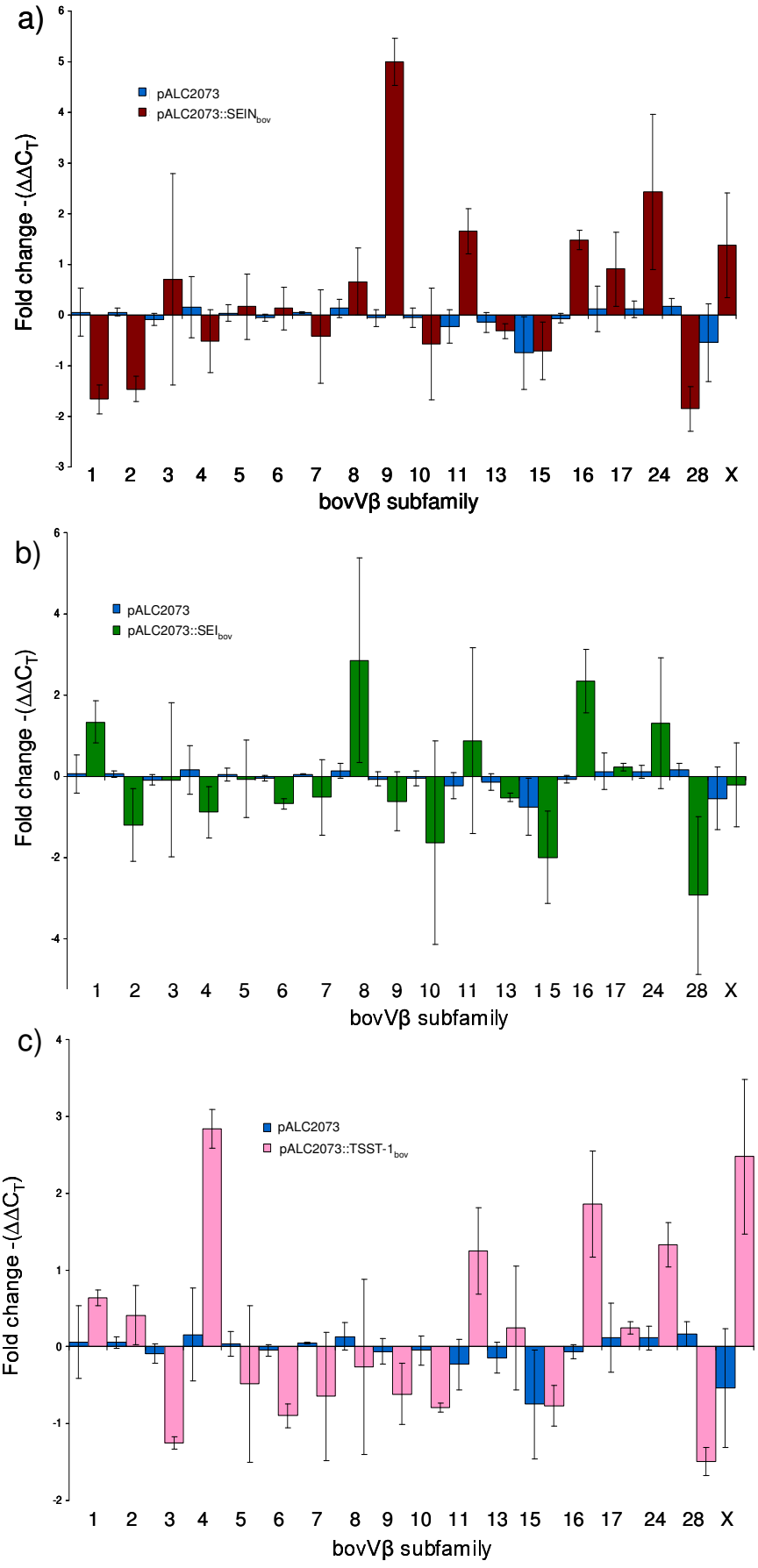


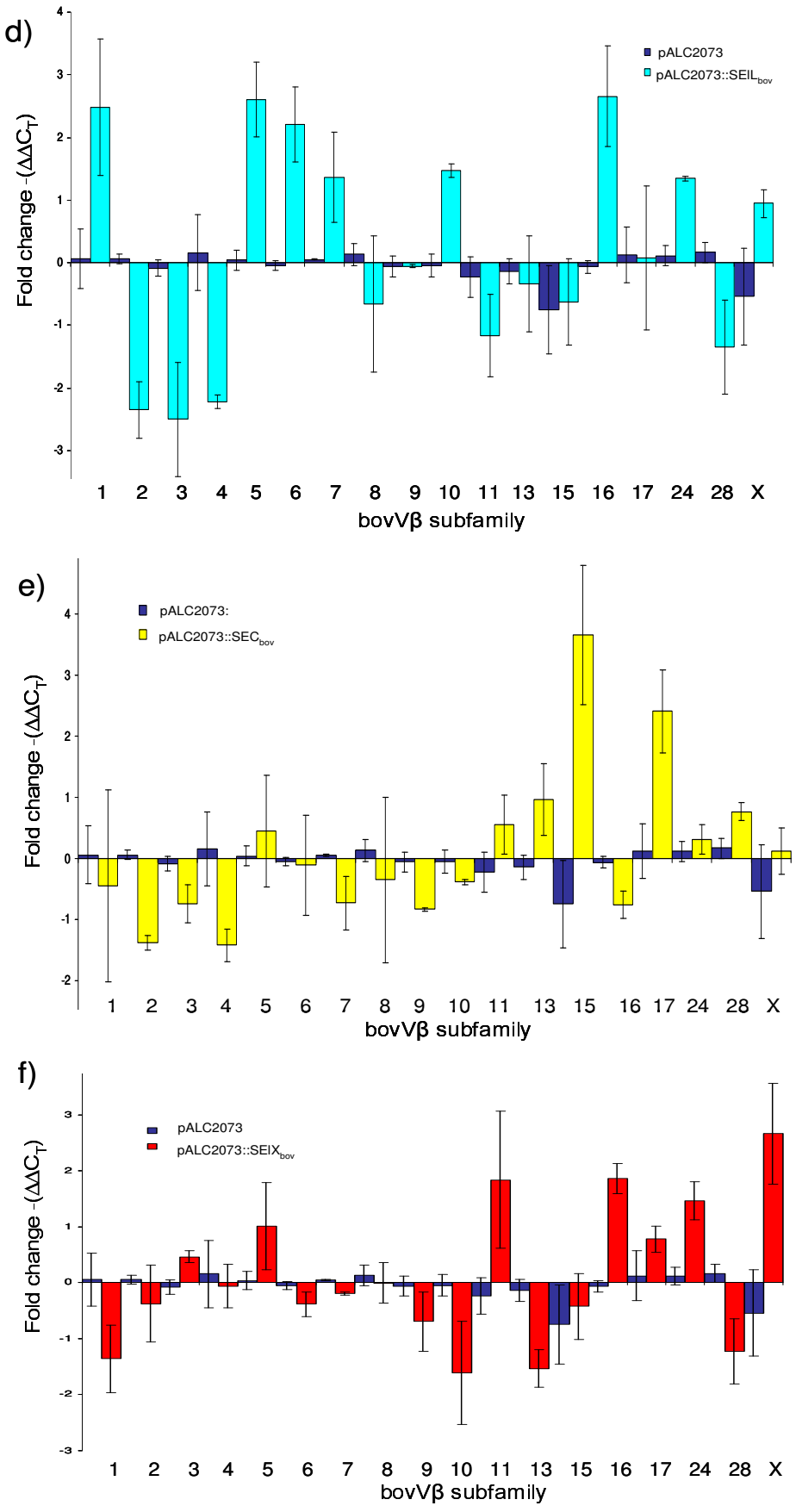
Figure 5.7: SEIZ$_{bov}$ has mitogenic activity for bovine T-cells, Bovine PBMC were labelled with CFSE prior to culture and stimulated for 4 d with **a)** medium only, or 10 ng/ml culture supernatants of **b)** RF122, **c)** RF122-7 (SAg-deficient except for *selzbov*, and **d)** RF122-8 (SAg-deficient). Results shown are from a single representative experiment that was performed at least twice.

subfamily specific primer pairs were designed within the bovVβ genes, with degenerate nucleotides used where sequence variation exists between subfamily members. In this study we have not confirmed the amplification of each individual subfamily member, but this could be achieved by cloning and sequencing of amplified qRT-PCR products. To increase specificity both primers were designed within the gene, rather than one primer within the gene and another within the β constant region as has been described for previous humVβ qRT-PCR studies (Ochsenreither *et al.*, 2008). In addition to β-actin, primers within the β constant region gene (Cβ) were used as an internal control for the number of T-cells.

Accordingly, for the first time we were able to comprehensively evaluate the response of 18 bovVβ subfamilies to stimulation with staphylococcal SAgs by qRT-PCR (Figure 5.8). SEC_{bov}, $SElL_{bov}$, $TSST\text{-}1_{bov}$, SEI_{bov}, $SElN_{bov}$, $SElX_{bov}$ and $SElY_{bov}$ were shown to induce Vβ-specific activation of bovine T-cells (Figure 5.8). Each of the RF122-encoded SAgs has a unique BovVβ activation profile which is summarised in Table 5.4, which is consistent with the effect of SAgs on humVβ cells (Choi *et al*., 1989).

Importantly, this study reveals that each of the bovVβ subfamilies are activated in response to at least one RF122-encoded SAg, such that RF122 has the potential to stimulate the entire bovVβ repertoire. BovVβ subfamilies activated by RF122-encoded SAgs are indicated on a phylogenetic tree of all functional TRBV gene sequences (Figure 5.9). It is also important to note that SaPIbov SAgs alone are able to activate 15 of 18 subfamilies. In comparison, *egc* SAgs activate only 8 of 18 subfamilies. Extensive duplication within the bovVβ repertoire has resulted in 9 multimember subgroups, the largest of which (bovVβ 1, 10 and 13) contain 23, 9 and 20 functional TRBV genes, respectively (Connelley *et al*., 2009, Elsik *et al*., 2009). Each of the SaPIbov encoded SAgs, SEC_{bov}, $SElL_{bov}$ and $TSST\text{-}1_{bov}$ and *egc* encoded SEI_{bov} can activate at least one of these large subfamilies (Figure 5.8). $SElL_{bov}$ activates both bovVβ 1 and 10 which is consistent with the large proportion of T-cells which are induced in reponse to stimulation (Figure 5.5).


a)
pALC2073
pALC2073::SEIN$_{bov}$
Fold change -(ΔΔC$_T$)
bovVβ subfamily
1 2 3 4 5 6 7 8 9 10 11 13 15 16 17 24 28 X
b)
pALC2073
pALC2073::SEI$_{bov}$
Fold change -(ΔΔC$_T$)
bovVβ subfamily
1 2 3 4 5 6 7 8 9 10 11 13 1 5 16 17 24 28 X
c)
pALC2073
pALC2073::TSST-1$_{bov}$
Fold change -(ΔΔC$_T$)
bovVβ subfamily
1 2 3 4 5 6 7 8 9 10 11 13 15 16 17 24 28 X


d)
pALC2073
pALC2073::SEIL$_{bov}$
Fold change -(ΔΔC$_T$)
4
3
2
1
0
-1
-2
-3
1 2 3 4 5 6 7 8 9 10 11 13 15 16 17 24 28 X
bovVβ subfamily
e)
pALC2073:
pALC2073::SEC$_{bov}$
Fold change -(ΔΔC$_T$)
4
3
2
1
0
-1
-2
1 2 3 4 5 6 7 8 9 10 11 13 15 16 17 24 28 X
bovVβ subfamily
f)
pALC2073
pALC2073::SEIX$_{bov}$
Fold change -(ΔΔC$_T$)
3
2
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0
-1
-2
-3
1 2 3 4 5 6 7 8 9 10 11 13 15 16 17 24 28 X
bovVβ subfamily

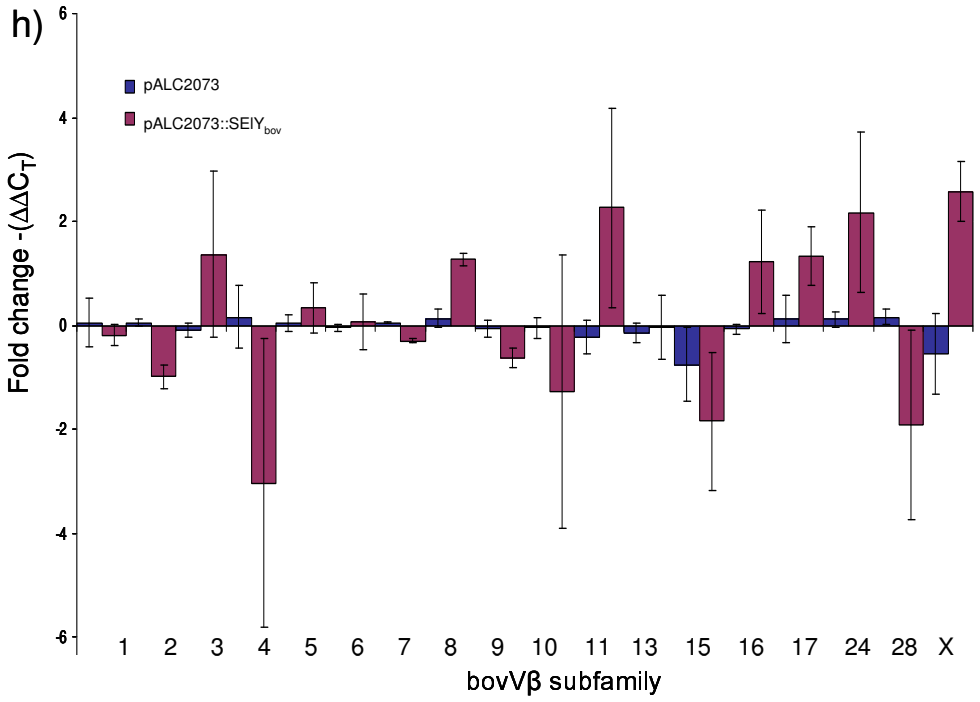


Figure 5.8: Vβ-dependent activation of bovine T-cell populations in response to stimulation with RF122-envoded SAgs. Relative fold change in bovVβ expression after 4 d stimulation with **a)** $SElN_{bov,}$ **b)** $SEI_{bov,}$ **c)** $TSST\text{-}1_{bov}$, **d)** SEC_{bov}, **e)** $SElL_{bov}$, **b)** $SElX_{bov}$ and **b)** $SElY_{bov.}$ Results shown are the means of triplicate measurements from 2 animals ± S.D.

Table 5.4: Activation of Vβ subfamilies in response to RF122-encoded SAgs.

SAg	Bovine Vβ [a, b, c]	SAg	Human Vβ [a, b, d, e]
TSST-1_{bov}	1, **2,** 4, 11, 16, 24, X	TSST-1	**2**
SElL$_{bov}$	**1, 5**, 6, **7**, 10, **16**, 24, X	SElL	**1, 5, 7**, **16**, 22, 23
SEC$_{bov}$	11, **13**, **15, 17**, 28	SEC$_{bov,}$	12, **13**, 14, **15, 17**, 20
SElN$_{bov}$	**8, 9**, 11, 16, **17,** 24, X	SElN	7, **8, 9, 17**
SEI$_{bov}$	**1**, 8, 16	SEI	**1**, 5, 6, 23
SElX$_{bov}$	3, 5, 11, 16, 17, 24, X	SElX$_{bov}$	1, 6, 18, 21
SElY$_{bov}$	3, 8, 11, 16, 17, 24, X	SElY$_{bov}$	NK
SElO$_{bov}$	N/A	SElO$_{bov}$	5, 7, 22
SElU$_{bov}$	N/A	SElU$_{bov}$	13, 14
SEG$_{bov}$	N/A	SEG$_{bov}$	3, 12, 13, 14, 15

[a] Vβ subfamilies were named according to the classification of Arden *et al* (Arden *et al*., 1995).

[b] Bovine and human Vβ subfamilies activated in response to the same SAg are highlighted in bold type.

[c] N/A, not applicable.

[d] NK, not known.

[e] HumVβ activation data has been compiled from Section 4.4 and the references (Deringer *et al*., 1997, Thomas *et al*., 2009, Seo *et al*., 2010).

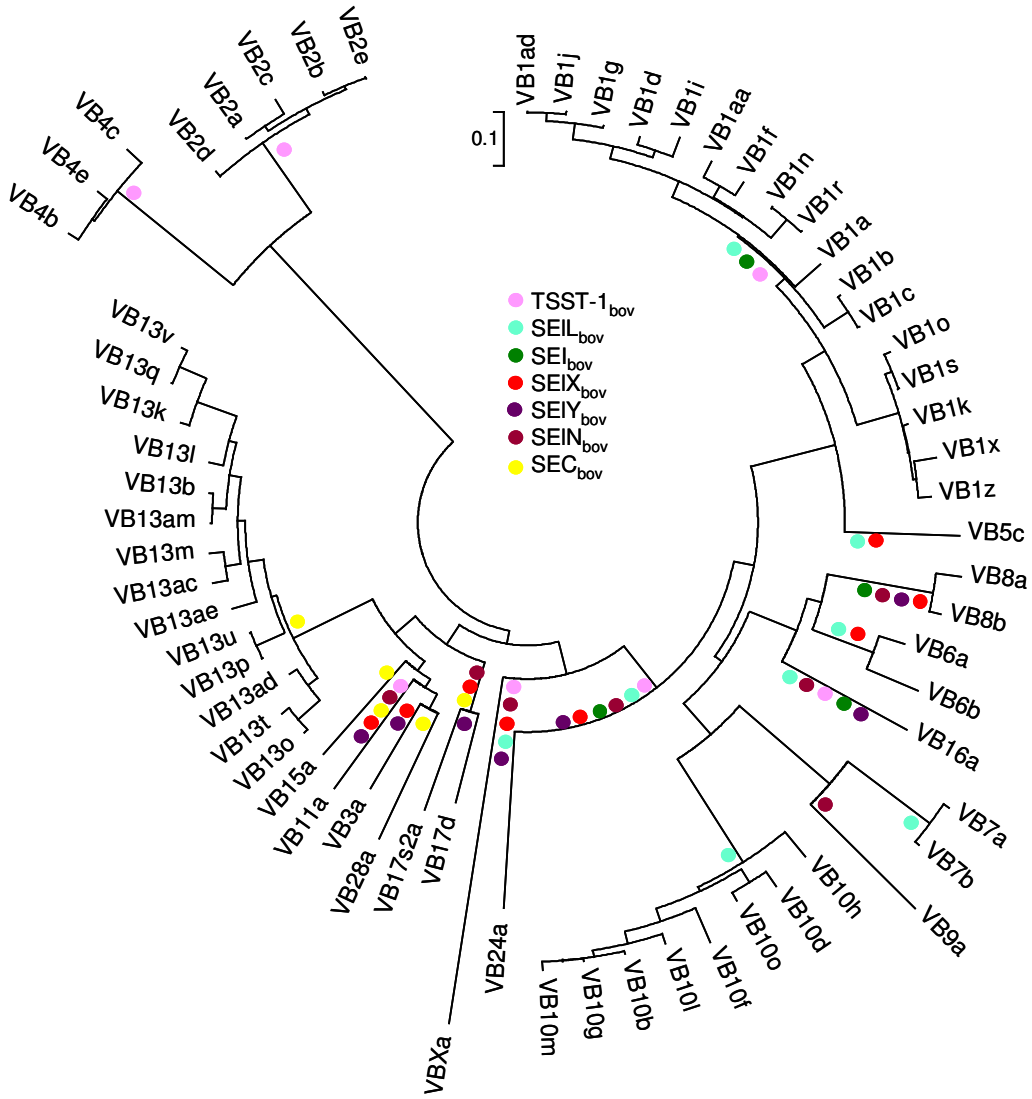


Figure 5.9: Phylogenetic analysis of bovine TCR Vβ gene sequences. Neighbour-joining tree based on all known TRBV gene sequences, constructed using MEGA from a ClustalW alignment of translated amino acid sequences. Coloured circles indicate activation of bovVβ subfamily in response to stimulation with RF122-encoded SAgs. At least 500 bootstrap trees were generated to examine the stability of the phylogenetic relationship.

5.4 Discussion

In this study we have investigated bovine T-cell activation in response to all SAgs encoded by a single bovine *S. aureus* strain, RF122. All 11 SAg genes encoded by RF122 were cloned into the pALC2073 plasmid facilitating controlled SAg gene expression in the SAg-deficient strain RF122-8α. Expression of SAgs in their natural *S. aureus* host should result in their native confirmation and avoids LPS contamination issues. Furthermore, functional activity may require the presence of co-factors made only by the native host.

Previously, only SEC_{bov} and TSST-1_{bov} had been shown to be mitogenic for bovine T-cells (Deringer *et al*., 1997, Marr *et al*., 1993, Fitzgerald *et al*., 2001a). In addition, in this work, we have previously described mitogenicity of rSElX (Figure 4.5). Here we have shown that in addition to $SElX_{bov}$, SEC_{bov} and TSST-1_{bov}, $SElL_{bov,}$ $SElN_{bov,}$ SEI_{bov} and $SElY_{bov}$ induced proliferation of bovine T-cells. SElU encoded by the *egc* is known to be mitogenic for human T-cells (Thomas *et al*., 2009, Thomas *et al*., 2006). In this study we have shown that RF122-8α supernatants containing the bovine variant $SElU_{bov,}$ are not mitogenic for bovine T-cells at total protein concentrations ranging from 10 pg/µl to 10 ng/ml after 4 d in culture. Thomas *et al* reported that proliferation of humVβ 13 and 14 was observed for SElU after 6 d at concentrations ranging from 1 µg/ml to 10 ng/ml. Previous studies have demonstrated maximal levels of Vβ specific T-cell proliferation in response to SAg are reached between 72 and 96 h after stimulation (Seo *et al*., 2007, Sriskandan *et al.*, 1996). Seo *et al* demonstrated that SEC1 induced non specific bovVβ specific T-cell proliferation after 96 h (Seo *et al*., 2007). Accordingly in this study we have measured T-cell activation after 96 h. We could not test concentrations above 10 ng/ml, as RF122-8α supernatants were toxic for bovine PBMC, which taken together with the shorter culture incubation time, could explain the lack of mitogenicity observed in response to stimulation with $SElU_{bov}$. Alternatively, it is possible that allelic variation between *selu1* and *selubov* is responsible for the functional difference observed. We were not able to confirm expression of *egc* SAgs $SElO_{bov}$ and SEG_{bov} in RF122-8α. Therefore further experiments will be required to investigate whether rSEG and rSElO are able to

activate bovine T-cells. It is noteworthy that SEG_{bov} is a truncated form of the characterised human SEG protein which implies the protein may not be functional.

Previously, we have carried out functional analysis of the novel SAg, SElX. Here we have demonstrated the mitogenic activity of novel SAgs $SElY_{bov}$ and $SElZ_{bov}$ for bovine T-cells. As a result these proteins can be described as SAgs and have been named in accordance with standard nomenclature (Lina *et al*., 2004). In addition to mitogenicity, for full functional characterisation of a SAg, further experiments to determine pyrogenicity and endotoxin enhancement will be required (Lina *et al*., 2004). Experiments with rSElZ and RF122-8α-Z induced in stationary phase will be required to confirm mitogenicity of SElZ. Of note, *selz* is phylogenetically related to *set* (Figure 3.2), a recently identified SAg which activates T-cells but is not Vβ specific (Ono *et al*., 2008), suggesting a T-cell activation mechanism which is distinct from that observed for previously characterised staphylococcal SAgs.

Vβ specific activation of human T-cells in response to staphylococcal SAgs has been well characterised (Seo *et al*., 2010, Thomas *et al*., 2009, Ono *et al*., 2008). This has largely been achieved by FACS analysis using a commercial kit, IOtest® Beta Mark (Immunotech Beckman Coulter) containing a panel of monoclonal antibodies (mAbs) against almost all human Vβ subfamilies, or using a recently developed qRT-PCR assay (Seo *et al*., 2010, Thomas *et al*., 2009, Ono *et al*., 2008). Unfortunately, no Vβ-specific monoclonal antibodies are available for cattle, and until recently TRBV gene information was limited. For the first time we have determined the unique bovVβ activation profile of bovine variants of previously characterised SAgs, $SEC_{bov,}$ $TSST\text{-}1_{bov}$, $SElL_{bov,}$ $SElN_{bov,}$ SEI_{bov}, and 2 novel SAgs $SElX_{bov}$ and $SElY_{bov}$ (Table 5.4). The bovVβ activation profiles determined for SEC_{bov} and $TSST\text{-}1_{bov}$ in this study are consistent with previous studies carried out with limited bovVβ subfamilies. Previously, Deringer *et al* examined the response of 5 bovVβ subfamilies to stimulation with SEC_{bov} by semi-quantitative PCR (Deringer *et al*., 1997). SEC_{bov} stimulated bovVβ 28 but not 1, 3, 4 or 7, which is consistent with our results. In addition, Fitzgerald *et al* investigated the response of the same 5 bovVβ subfamilies to stimulation with stationary phase culture supernatants of RF122 and sequential mutants RF122-1 (Δ*tstbov*) and RF122-2 (Δ*tstbov*Δ*secbov*). RF122 supernatant activated bovVβ 1, 4, 7, and 28 but

not VB3 (Fitzgerald *et al*., 2001a). RF122-1 was unable to activate bovVβ 4, which was attributed to the deletion of *tstbov* in this strain. Similarly, RF122-2 was unable to activate bovVβ 28, due to the deletion of *secbov* (Fitzgerald *et al*., 2001a). Here we have demonstrated that TSST-1_{bov} is the only RF122-encoded SAg which can activate bovVβ 4, and SEC_{bov} is the only SAg that can activate bovVβ 28 (Figure 5.8). Previously, RF122-3 was able to activate bovVβ 1 and 7, here we have shown that $SElL_{bov}$ activates both of these subfamilies, and is transcribed at high levels in stationary phase (Figure 3.3) (Fitzgerald *et al*., 2001a).

We have demonstrated that RF122 has the potential to stimulate the entire bovVβ repertoire by expression of SAgs (Figure 5.9). It has been shown previously that all humVβ subfamilies are activated by at least one SAg (Thomas *et al*., 2009). However activation of all humVβ by the SAgs encoded by a single strain has not been reported. Our data also indicates that some bovVβ subfamilies can be activated by multiple SAgs, for example, Vβ 24 is activated by 6 SAgs, and Vβ 6, 11 and X are activated by 5 SAgs (Figure 5.9). This apparent functional redundancy implies an important role in pathogenesis for activation of these subgroups. A similar redundancy has been observed in the humVβ response to SAgs, Vβ 5 and 14, and Vβ 18 and 21 were targeted by 5 and 6 different SAgs respectively (Thomas *et al*., 2009). It has been proposed that humVβ profiles could be used diagnostically to indicate SAg exposure during some staphylococcal diseases, such as TSS (Ferry *et al.*, 2008). However the observed redundancy in SAg activation of specific bovVβ subfamilies suggests this would not be feasible as a diagnostic test for bovine mastitis infections.

For the first time we have been able to compare the human and bovine Vβ activation in response to staphylococcal SAgs, which has revealed that bovVβ activation is distinct from humVβ activation. Our data reveals some similarities, but also marked differences in activation which suggests host species adaptation. (Table 5.4) (Seo *et al*., 2010, Thomas *et al*., 2009). There are a number of human and bovine subfamilies activated in common, by each SAg (Table 5.4). The differences observed are in part due to activation of a bovine or human Vβ subgroup for which there is no orthologous subgroup, such as the activation of humVβ 12, 14, 20, 22, and 23, and bovVβ 10, 28 and X (Connelley *et al*., 2009). However there are a number of subfamilies from one host which have been activated, but the

orthologous subgroup from the other is not. Of note, SEC_{bov} activates bovVβ 11 but not humVβ 11. Similarly, $SElL_{bov}$ activates bovVβ 6 and 24, TSST-1_{bov} activates bovVβ 1, 4, 11, 16, 24, SEI_{bov} activates bovVβ 8 and 16 and humVβ 5 and 6, and $SElN_{bov}$ activates bovVβ 11, 16, 24 and humVβ 7, but not the equivalent human or bovine subgroups. It is important to note that with the exception of SEC_{bov}, human Vβ profiles described here are in response to stimulation with SAgs encoded by human *S. aureus* strains (Seo *et al*., 2010, Thomas *et al*., 2009). Allelic variants of *sell* differ by one synonymous nucleotide, therefore it can be inferred that $SElL_{bov}$ and SElL are identical. Translated amino acid sequences of TSST-1_{bov}, $SElN_{bov}$ and SEI_{bov}, have 97, 94 and 95% identity with human variants respectively. Host specific SAgs variants have been shown to activate distinct Vβ profiles, SEC1 and SEC_{ov} induced proliferation of bovVβ 26 and bovVβ 28 whereas SEC_{bov} induced bovVβ 28 only (Deringer *et al*., 1997), therefore this must be considered when comparing the bovine and human Vβ activation by TSST-1, SEI and SElN.

Chapter 6

Experimental intra-mammary infection of dairy cattle with *S. aureus* RF122 and a superantigen-deficient derivative

6.1 Introduction

Bovine mastitis caused by *S. aureus* infection is typically chronic in nature with the host often unable to eliminate the organism (Ferens & Bohach, 2000, Sol *et al.*, 2000). The factors responsible for this persistence in the host are not well understood. It has been proposed that SAgs may contribute to persistence by modulation of the bovine immune response. In the current study, analysis of the genome sequence of bovine clinical isolate RF122 revealed an array of SAg genes. Here we have demonstrated that RF122-encoded SAgs are expressed *in vitro* and have superantigenic activity for bovine T-cells. To investigate the effect of SAgs expressed during *S. aureus* disease pathogenesis *in vivo*, targeted disruption of genes leading to loss of function will be carried out to allow the contribution of individual loci to be determined.

The aim of this component of the study was to investigate the role of SAgs in *S. aureus* pathogenesis and persistence of disease by:

- Constructing a SAg-deficient derivative of RF122 by allele replacement.

- Experimental infection of dairy cattle with wild type and SAg-deficient strains.

6.2 Materials and Methods

6.2.1 Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are indicated in Table 6.1. Media was supplemented where appropriate with erythromycin (Fisher Scientific, UK) at 10 μg/ml (*S. aureus*) or 200 μg/ml (*Escherichia coli*), 50 μg/ml ampicillin (Sigma-Aldrich, UK) and 150 μg/ml X-gal (Melford, UK).

6.2.2 Construction of allele replacement plasmids

pMAD gene deletion constructs were generated by overlapping PCR as outlined in Figure 6.1. PCR products of approximately 500 bp flanking the left (A and B) and right (C and D) region of the gene of interest were amplified with primers listed in Table 6.2. PCR amplification with primers A and D was carried out with approximately 100 ng of purified AB and CD template DNA to produce a single spliced amplicon. Sequence complementary to primer B was incorporated into the CD fragment by Primer C, which allows the products of the first PCR to anneal. AD products were then purified and cloned into the Strataclone pSC-B plasmid (Stratagene) according to the manufacturer's instructions. Expected sizes of AB, CD and AD amplicons and the regions to be deleted associated with each plasmid constructed in this study are shown in Table 6.2. pSC-B plasmid containing the AD insert was restriction digested with *Eco*R1 for 2 h at 37 °C, or digested sequentially with *Sma*I at 25 °C overnight and *Sal*I at 37 °C for 6 h. The AD fragment was then purified, ligated into the dephosphorylated gene replacement plasmid, pMAD, and transformed into *E. coli* strain DH5α. The resulting pMAD deletion construct was isolated from *E. coli* and transformed by electroporation into the *S. aureus* strain of interest.

Table 6.1: Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source/ Reference
S. aureus		
RF122	Wild type bovine mastitis	(JR Fitzgerald, 1997)
RF122t	Transducable variant of RF122	(Fitzgerald *et al*., 2001a)
RF122-1	*tst*::Tc^r	(Fitzgerald *et al*., 2001a)
RF122-3	*tst*::Tc^r Δ*sec*	This study
RF122-4	*tst*::Tc^r Δ*sec*Δ*sell*	This study
RF122-5	*tst*::Tc^r Δ*sec*Δ*sell*Δ*egc*	This study
RF122-6	*tst*::Tc^rΔ*sec*Δ*sell*Δ*egc*Δ*selx*	This study
RF122-7	*tst*::Tc^rΔ*sec*Δ*sell*Δ*egc*Δ*selx*Δ*sely*	This study
RF122-8	*tst*::Tc^rΔ*sec*Δ*sell*Δ*egc*Δ*selx*Δ*sely* Δ*selz*	This study
RF122t Δα	Δ*hla*	This study
RF122-8Δα	*tst*::Tc^rΔ*sec*Δ*sell*Δ*egc*Δ*selx*Δ*sely*Δ*selz*Δ*hla*	This study
RN4220	Restriction/modification$^-$ derivative of NTCC8325	(Kreiswirth *et al*., 1983)
Newman	Human isolate	(Duthie, 1952)
E. coli		
TOP10	Chemically competent	Invitrogen
Strataclone SoloPack™	*lacZΔM15* mutation, *endA*, *recA*-deficient	Stratagene

Strain or plasmid	Description	Source/ Reference
Plasmids		
pSC-B	Contains *lacZ*' α-complementation cassette	Stratagene
pMAD	Thermosensitive gene replacement plasmid, pE194 derivative	(Arnaud *et al.*, 2004)
pMAD:*secbov*	*secbov* deletion construct	This study
pMAD:*sellbov*	*sellbov* deletion construct	This study
pMAD:*egc*	*egc* deletion construct	This study
pMAD:*selxbov*	*selxbov* deletion construct	This study
pMAD:*selybov*	*selybov* deletion construct	This study
pMAD:*selzbov*	*selzbov* deletion construct	This study
pMAD:*hla*	*hla* deletion construct	This study

Table 6.2: Primers used to obtain and verify pMAD gene deletion constructs.

Primer	Sequence (5´ - 3´) [a]
*secbov*A	ATGAATTCCTGTGGATTTAGAAATAAGG
*secbov*B	CCAACATTCCCAAGAAGTATC
*secbov*C	GATACTTCTTCTTGGGAATGTTGGAAGAATGGATAATGTTAATCC
*secbov*D	TTATCCATGGCAAGCATCAAAC
*secbov*E	GTCATGTTTCGGTTGATAGG
*secbov*Z	ATGGCGGTGTTACTAAAGC
Up*sec*F	GCAGGTACTTCGGTACTTGCCTAT
Up*sec*R	GGAGAAACAGAGGATTTCTAAGCATC
*sec*probe-F	TGAAGGAAACCACTTTGATAATGGG
*sec*probe-R	AGATTGGTCAAACTTATCTCCTGGT
*sellbov*A	GATATATTTGAAAGGTAAGTACTTCG
*sellbov*B	AGTGTAGTATTCCATATGAATGATGGT
*sellbov*C	ACCATCATTCATATGGAATACTACACTATACAAAAGGTTATAGGAAGAGTT
*sellbov*D	CAATTTCTACAGATATGACTCCC
*sellbov*Z	TGTACAAATGGACTTAAGATATAGCG
*egc*A	TCTTGATACGTATTTGACACTTGC
*egc*B	AGCTATACGAGTTTGATGGTTCTG
*egc*C	CAGAACCATCAAACTCGTATAGCTAACTAAGCGACTCAGATAATAGAC
*egc*D	AGAGTTGTTACAGTCGCTACACC
*egc*E	AGCACAAACTGTAGCAGAACATGAG
*egc*Z	GTATATAGTAGGAATGAACTATATAGCC
Up*egc*-F	GAAGAAGTATTTGAATTCTTATATGACC
Up*egc*-R	CATCATTTACAATTATTAACATGATAGG
*selxbov*A	TGTCGATGCTATGGATAGTGAGG
*selxbov*B	TAATTACCTCCTTGATGTAAAGC

Primer	**Sequence (5´ - 3´)** [a]
*selxbov*C	GCTTTACATCAAGGAGGTAATTATATCGCTAATACTTTGAAAG TTAGG
*selxbov*D	TCAAATGTAGCAGTATACATTAATTGCG
*selxbov*E	ATCTCAGTTGTCTCTTTGATAGTGC
*selxbov*Z	TTACTCTTCATGTGTCACTTCATTTCG
*selx*probe-F	AATCTAATAAAGCTAGGAATTGGG
*selx*probe-R	TGTAACTCTTTGTGCGACTCTAATG
Up*selx*-F	GTGTATCTTAATTTCATATCTATAGTTGC
Up*selx*-R	AAGCAATGCAAGAGCATGTAGG
*selybov*A	ACTTGAAGGTATGTATGTTACACG
*selybov*B	TCTAGCTTCCAACAAAGAATAGC
*selybov*C	GCTATTCTTTGTTGGAAGCTAGAGGTAATGAAACAGTTGAAT CAGC
*selybov*D	AAGAACCACGCGAACACACAAGC
*selybov*E	AGATTATTAGGTAAGGCATAAATAGC
*selybov*Z	TCAGCTTACAATAATGTGCAAGTTGG
*sely*probe-F	GAAAGAGTAACACTGTCATTCTCG
*sely*probe-R	TTGAGTCATGACTTCATTGTTCC
Up*sely*F	ATTCTAATGTTTACGTTCTTCATAGC
Up*sely*R	CACAAATTGCAATTACTGTTATACG
*selzbov*A	AAGGTGACAATCCTGAAATCAC
*selzbov*B	CAGATCATCCTTTCTCATTTAAGATT
*selzbov*C	AATCTTAAATGAGAAAGGATGATCTGGTTCCAACATAAATGCG
*selzbov*D	TTATGGCTCAGGTTCAGTTGGT
*selzbov*E	TAGGACGATTGGAACTTGTGAG
*selzbov*Z	ATATGAAGATGCTGTAGATTATAACC
*hla*A	ATTCATCATTAGAAGCTAACCTATACTC
*hla*B	GATTTGAGGAAACAATAATCAATATGTC
*hla*C	GACATATTGATTATTGTTTCCTCAAATCTGTAAATTGTTTGTT CAT
*hla*D	TCAGAACCATAGTTGACATGAGC

Primer	**Sequence (5´ - 3´)** [a]
*hla*E	GAAATTTTATAGCCTGATTCAGACTC
hlaZ	TTATCTAATTTTCATTTGCTTTACATG
pMAD-2m	AAGCGAGAATCATAATGGG
pMAD-3c	CTTGCTCCAACTGAAAATCCC

[a], Complementary regions of allele replacement primers are underlined.

Table 6.3: Generation of pMAD gene deletion constructs. Size of AB and CD products, spliced AD fragments and deletion obtained.

Deletion target	AB (bps)	CD (bps)	AD (bps)	Deletion (bps)
secbov	512	506	1018	825
sellbov	285	320	605	685
egc	614	641	1255	5319
selxbov	532	478	1010	674
selybov	555	564	1119	668
selzbov	546	480	1026	673
hla	604	638	1242	988

6.2.3 Allelle replacement mutagenesis by temperature shifting with enrichment

S. aureus containing the pMAD deletion construct of interest was shaken overnight at 30 °C in 2 ml TSB containing erythromycin. To select for integration of the plasmid into the chromosome through homologous recombination (Figure 6.1), dilutions of this culture between 10^{-2} and 10^{-8} were incubated on TSA containing erythromycin at a non-permissive temperature for plasmid replication, 45 °C. Colonies which grew at this temperature were sub-cultured to single colonies on TSA containing erythromycin at 45 °C to ensure a pure culture, and checked by PCR for single cross over integration. To generate double cross-over excisants, integrants were then grown in TSB without antibiotic at 30 °C for 24 h. 10-fold serial dilutions were plated onto TSA containing X-gal and incubated on TSA at 30 °C overnight. Colonies were then screened for sensitivity on erythromycin plates (inferring loss of the plasmid) and screened for WT or mutated forms of the genes by colony PCR with primers upstream (E) and downstream (Z) of the gene of interest (GOI). The resulting mutant strain which has lost the GOI was further verified by PCR analysis for no amplification with primers within the deleted region or with pMAD MCS primers. The mutant strains were also sequenced using primers upstream (E) and downstream (Z) of the GOI to confirm the predicted deletion event.

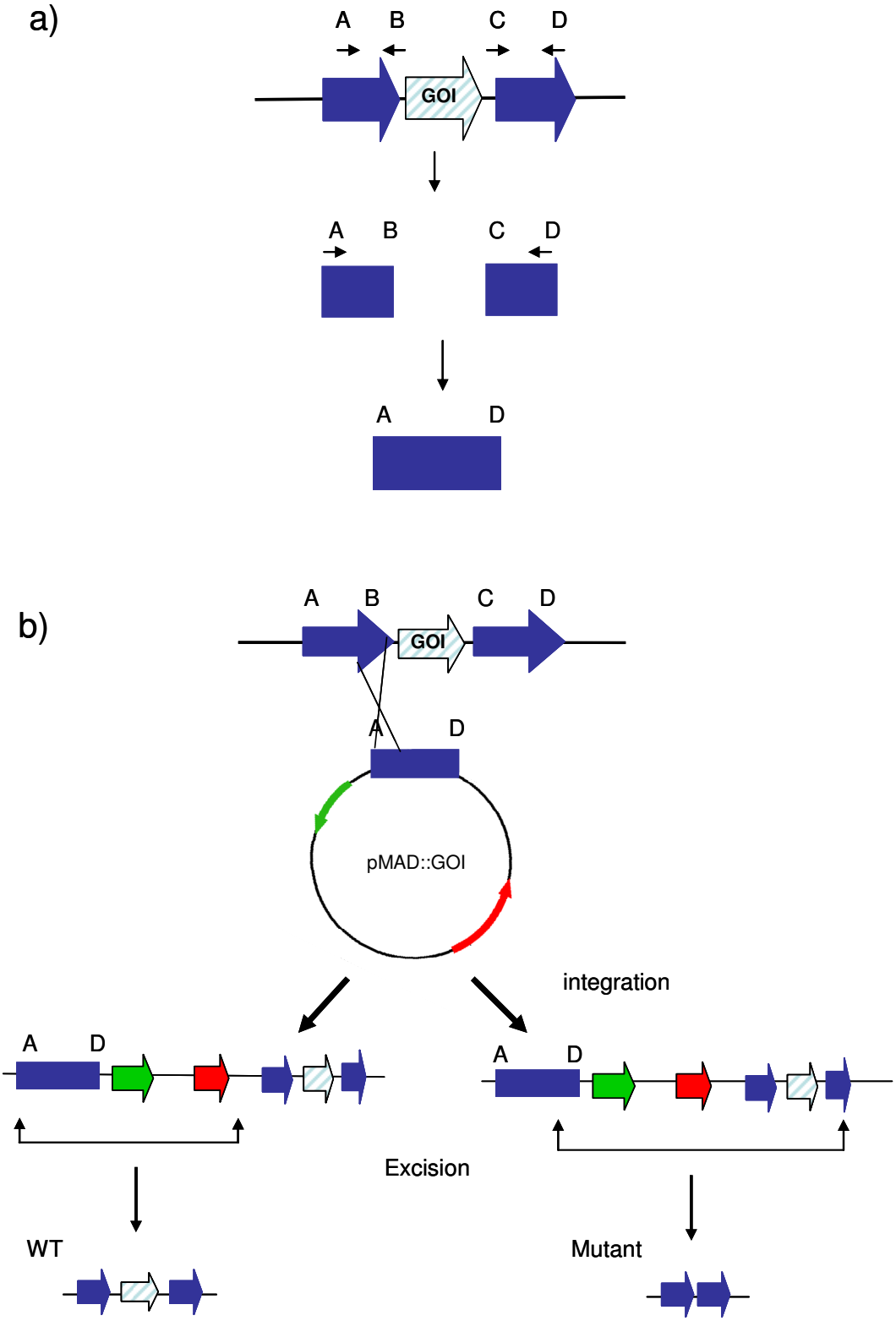

a)
A
B
C
D
GOI
A
B
C
D
A
D
b)
A
B
C
D
GOI
A
D
pMAD::GOI
integration
A
D
A
D
Excision
WT
Mutant

Figure 6.1: Schematic diagram of the procedure used to obtain gene replacement by recombination using the pMAD plasmid. **a)** PCR products of approximately 500 bp flanking the left (A and B) and right (C and D) of the gene of interest were amplified. Primer B is complementary to primer C which allows the products of the first PCR to anneal at the region of overlap during a second PCR with primers A and D. The AD product is then cloned into the pMAD plasmid.
b) Areas labelled AB and CD represent DNA sequences located upstream and downstream of the gene of interest. The crossed lines indicate a crossover event. Single crossover integration via homologous recombination can occur at either region. The co-integrate then undergoes a second recombination event with excision of the pMAD plasmid, resulting in either recreation of the wild type or a gene deletion mutant.

6.2.4 Southern Hybridisation

2 μg genomic DNA was digested with *Hind*III restriction endonuclease overnight at 37 °C and analysed by agarose gel electrophoresis in a 0.8% (w/v) agarose gel. The gel was depurinated in 0.25 M HCl (Fisher Scientific, UK) for 10 min, immersed in denaturation solution containing 0.5 M NaOH (Fisher Scientific, UK) and 1.5 M NaCl (Melford, UK) for 1 h, followed by neutralisation solution (0.5 M TrisHCL, 1.5 M NaCl, pH 7.5) for 1 h. DNA fragments were transferred by alkaline capillary blotting onto a nylon membrane (Amersham, GE Healthcare). Labelling of the probes and DNA hybridisation was performed according to the protocol supplied with the ECL Direct nucleic acid labelling and detection system (Amersham, GE Healthcare).

6.2.5 Extraction of *S. aureus* secreted and CWA proteins

Secreted and CWA proteins were extracted from *S. aureus* mid-exponential (3 h) and stationary phase (12 h) cultures grown in BHI. Cells were centrifuged at 4000 x g and supernatant fractions containing secreted proteins were removed and concentrated with Amicon Ultra-15 Centrifugal Filter units with a 10 kDa MWCO as described in the manufacturers instructions (Millipore, Watford, UK). Cell-wall associated proteins were extracted from pelleted cells by washing with 1 ml PBS (Oxoid, Cambridge, UK), resuspension in 1 ml lysis buffer (50 mM TrisHCl, 20 mM $MgCl_2$, 30% Raffinose (Fluka, UK), adjusted to pH 7.5) containing 200 μg/ml Lysostaphin (AMBI products LLC, NY, USA) and protease inhibitors (Sigma-Aldrich, UK) and incubation at 37 °C for 20 min. Samples were centrifuged at 6000 x g for 20 min and the CWA proteins were recovered from the supernatant fraction. Proteins were seperated by SDS-PAGE as described in General Methods, and stained overnight at room temperature with Coomassie Blue (Severn Biotech).

6.2.6 Haemolytic activity of *S. aureus*

Overnight cultures of *S. aureus* strains grown in TSB were centrifuged at 4000 x g and supernatants obtained to assess the haemolytic activity of *S. aureus* cuture supernatants by titration. Doubling dilutions of the supernatants were mixed with equal volumes rabbit erythrocytes. Erythrocytes were obtained from blood by washing 3 times with 2 x PBS for 10 min at 1500 x g and 4 °C and re-suspended as

2% (w/v) packed cell volume in PBS in round bottomed microtitre plates (Corning incorporated). End points were assessed after incubation at 37 °C for 30 min and overnight incubation at 4 °C. The reciprocal of the dilution in the last well showing 50% lysis is the haemolytic titre for each strain.

6.2.7 Growth curve of *S. aureus* strains

S. aureus strains were cultured overnight in 5 ml Brain–Heart Infusion (BHI) broth (Oxoid Ltd., Basingstoke, UK) in triplicate. After 12 h strains were sub-cultured at a dilution of 1/100 into 30 ml fresh BHI broth in 250 ml Erlenmeyer flasks and placed in a shaking incubator at 37 °C and 200 rpm. Absorbance readings were measured at 600 nm (OD_{600}) using a spectrophotometer (Cecil Aurius CE2021, Thistle Scientific Ltd., Glasgow, UK) over a period of 12 h and a growth curve was determined.

6.2.8 Experimental infection of dairy cattle

Lactating Holstein cows from the University of Idaho dairy herd were assigned to 2 experimental groups, positive control and experimental. Positive control group received intramammary instillations of the wild type strain *S. aureus* RF122t to one udder quarter, and the experimental group received the SAg-deficient mutant RF122-8 (Figure 6.2). PBS was instilled into an udder quarter of the other udder half as a negative control of infection. Bacterial suspensions for intramammary instillations were prepared in 10 ml PBS and contained 2, 5 or 10 CFU (colony forming units). Inocula were prepared by serial dilution and verified by viable counts. Milk samples were obtained daily from each mammary quarter using a specially adapted quarter milking apparatus (Figure 6.2). Somatic cell count (SCC) of milk was determined by measuring DNA content, using the membrane filter technique developed by Bremel *et al* (Bremel, 1980). In brief, 2.5 ml of milk was mixed with 20 ml of a warm diluent (40 °C) containing 0.1% vol/vol of Triton X-100 (Sigma, St. Louis, MO) and 4.5 g/L trisodium EDTA in PBS, and filtered through a cellulose acetate membrane filter of 3 to 5 μm pore size to retain somatic cells. The membrane was rinsed with 2 ml of PBS to remove detergent and individual filters were placed in glass tubes containing 5 ml of colour reagent (1:2:1 parts of 5 M HCl, water, and 0.06 % indole). Filters were incubated at 90 °C for 25

min, and colour development was quenched by immersion in cold water. The OD of the samples was measured at 490 nm using a spectrophotometer. A cell-free milk sample was processed to obtain a blank sample (Ward & Schultz, 1973).

Bacterial counts were determined by inoculating serially-diluted squirt milks directly onto blood agar plate. Intramammary infection (IMI) was considered established when 2 daily cultures were positive for *S. aureus.* Elevation of body temperature >105 °F indicated a high risk for loss of the animals (University of Idaho animal use guidelines), therefore experiments were stopped by treatment with anti-inflammatory drug (Benamin), aspirin, and penicillin G. Intramammary instillation of cattle and SCC measurements were performed by Brent Hatch and Keun Seok Seo (University of Idaho, USA).

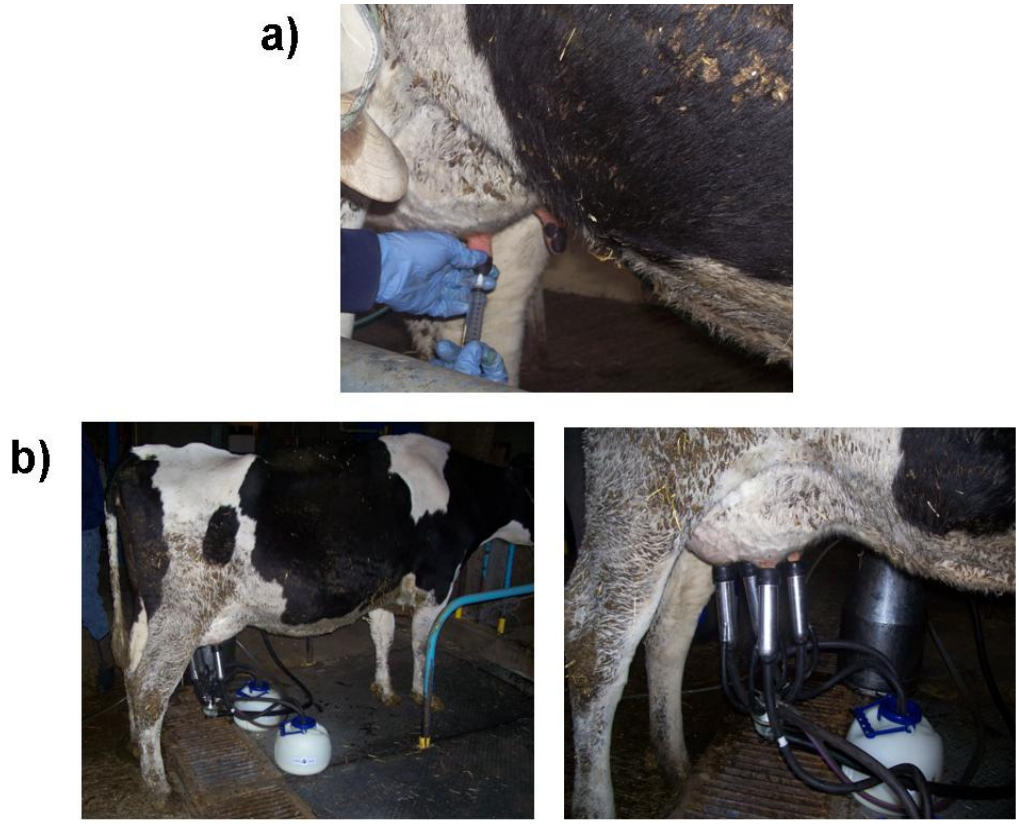


Figure 6.2: Experimental infection of dairy cows. **a)** Direct intra-mammary inoculation of the teat. **b)** Milk was collected using adapted quarter milking equipment.

6.3 Results

6.3.1 Construction of a SAg-deficient derivative of RF122

S. aureus RF122-1, a *tstbov*-deficient derivative of the bovine clinical isolate RF122 had been constructed previously by disruption of the *tst* gene with a tetracycline resistance cassette (Fitzgerald *et al*., 2001a). To delete all the remaining SAg genes encoded by RF122-1 by sequential allelic replacement, pMAD constructs, pMAD:*secbov*, pMAD:*sellbov*, pMAD:*egc*, pMAD:*selxbov*, pMAD:*selybov* and pMAD:*selzbov* were generated. Spliced AD fragments of regions flanking *secbov*, *sellbov*, *egc*, *selxbov*, and *selybov* were cloned into the *Eco*RI site of pMAD. There is an *Eco*RI site in the sequence downstream of *selz* (CD fragment). Accordingly the spliced AD fragment for *selzbov* was cloned between the *Sal*I and *Sma*I sites of pMAD. pMAD:*secbov*, pMAD:*sellbov*, pMAD:*egc*, pMAD:*selxbov* and pMAD:*selybov* were digested with *Eco*RI to confirm the correct size of the cloned AD fragments (Figure 6.3), and PCR analysis was used to confirm the correct size of the cloned AD fragment of pMAD:*selzbov,* 1026 bp (Figure 6.3).

Initially the pMAD:*secbov* plasmid was transformed into RF122-1. Integration of the plasmid into the RF122-1 chromosome was selected for on TSA containing erythromycin at a non-permissive temperature for plasmid replication, 45 °C. Colonies which grew at this temperature were checked for single cross over integration by PCR with a chromosomal primer downstream of the AD region, *secbov*Z and either pMAD MCS primer pMAD-2m or pMAD-3c. To generate double cross over excisants, integrants were then grown in TSB without antibiotic at 30 °C for 24 h. Colonies obtained after incubation on TSA at 30 °C overnight were then checked for sensitivity on erythromycin plates (loss of the plasmid) and screened for wild type or mutated forms of the genes by colony PCR.

To confirm that allelic exchange had been successful, and a mutated form of the *secbov* gene was inherited, PCR amplication with primers *secbov*E and *sellbov*Z across the deleted gene revealed that the size of the product was consistent with the presence of the mutant allele. The EZ region amplified from strain RF122-1 was 3070 bp, whereas a region of 2245 bp was amplified from the final RF122-3

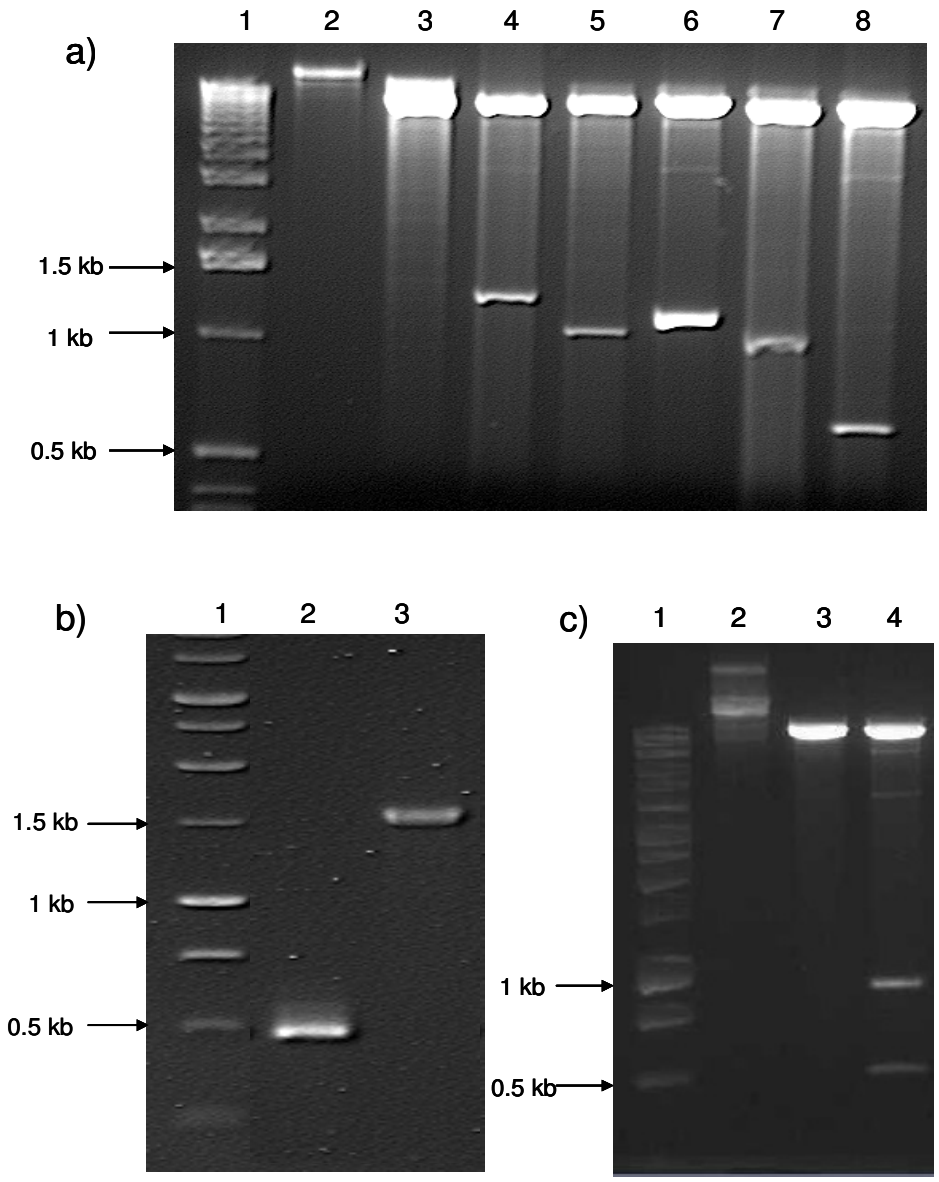


Figure 6.3: Verification of pMAD deletion plasmids. a) *Eco*R1 restriction digests of pMAD deletion constructs to confirm the size of the AD inserts. Lane 1, 1kb+ ladder; lane 2, uncut pMAD; lane 3, *Eco*RI digested pMAD; lane 4, pMAD:*egc*; lane 5, pMAD:*selybov*; lane 6, pMAD:*selxbov*; lane 7, pMAD:*secbov*; lane 8, pMAD:*sellbov*. **b)** PCR analysis of pMAD:*selzbov* with pMAD multiple cloning site primers 2m and 3c. Lane 1, 1kb+ ladder; lane 2, pMAD; and lane 3, pMAD:*selz*. **c)** *Eco*R1 restriction digests of pMAD:*hla* deletion constructs. Lane 1, 1kb+ ladder; lane 2, uncut pMAD; lane 3, *Eco*RI digested pMAD; lane 4, pMAD:*hla*.

(Δ*tst*Δ*secbov*) mutant strain indicating a deletion of 825 bp from the *secbov* gene (Figure 6.4).

In the same way, each SAg gene was deleted sequentially in this strain resulting in the final SAg-deficient derivative RF122-8 (Figure 6.5). Allelic replacement of *sellbov* was carried out in RF122-3 using pMAD:*sellbov,* generating RF122-4 which is deficient in all SaPIbov SAg genes, *tst, secbov* and *sellbov* (Figure 6.4). Allele replacement of the entire 5.3 kb *egc* region in RF122-4 was then carried out using pMAD:*egc* resulting in RF122-5, which is deficient in SaPIbov and *egc* SAg genes. PCR amplification with *egc*E and *egc*Z revealed the region in RF122 to be the expected size of 6898 bp and RF122-5 was 1579 bp, consistent with a 5319 bp deletion of the *egc* region (Figure 6.4). Similarly pMAD:*selxbov* was used to delete putative SAg gene *selxbov* in RF122-5, producing RF122-6. PCR amplification with *selxbov*E and *selxbov*Z revealed bands of the expected sizes, 2108 bp for RF122 and 1440 bp for RF122-6, representing a 668 bp deletion of *selx* (Figure 6.4). In turn, *selybov* was deleted in RF122-6 using pMAD:*selybov,* creating RF122-7. PCR amplification using *selybov*E and *selybov*Z showed the region spanning the gene to be 2043 bp in RF122 and 1369 bp in RF122-7, demonstrating that a 674 bp deletion of *selybov* has been introduced (Figure 6.4). Finally, allele replacement of *selz* was carried out in RF122-7 with pMAD:*selz*. PCR analysis with *selzbov*E and *selzbov*Z across the gene produced bands of the expected sizes, 1966 bp for RF122 and 1296 bp for RF122-8, indicating a deletion of 670 bp has occurred (Figure 6.4). The resulting SAg-deficient strain RF122-8 contained deletions in each of the SaPIbov, *egc* and putative SAg genes (Figure 6.5).

Further confirmation of each deletion event was provided by no amplification with primers outside and within the deleted regions (results not shown), sequencing across the deleted regions, and Southern blot analysis of the wild type and mutant strains with probes specific for regions of DNA upstream or downstream of the deleted regions (Figure 6.6). The size differences between hybridising *Hind*III-digested fragments of genomic DNA from each strain with the probes for each region was evaluated. In each case the hybridising fragment of the

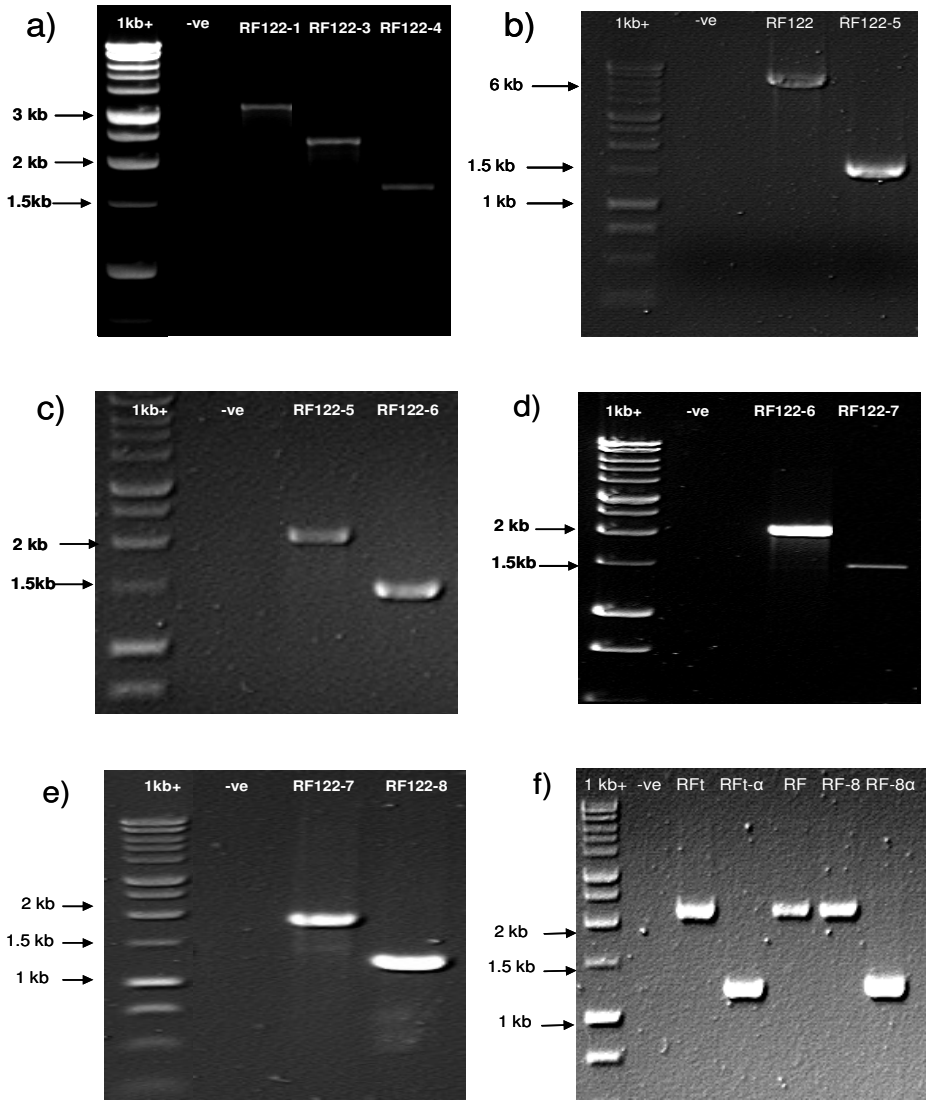


Figure 6.4: PCR verification of deleted SAg genes. a) PCR amplification of the SaPIbov region in RF122 and derivatives using primers *secbov*E and *sellbov*Z. PCR analysis with primers (E and Z) spanning the deleted region of each gene, **b)** *egc*, **c)** *selx* **d)** *sely,* **e)** *selz,* **f)** *hla*.

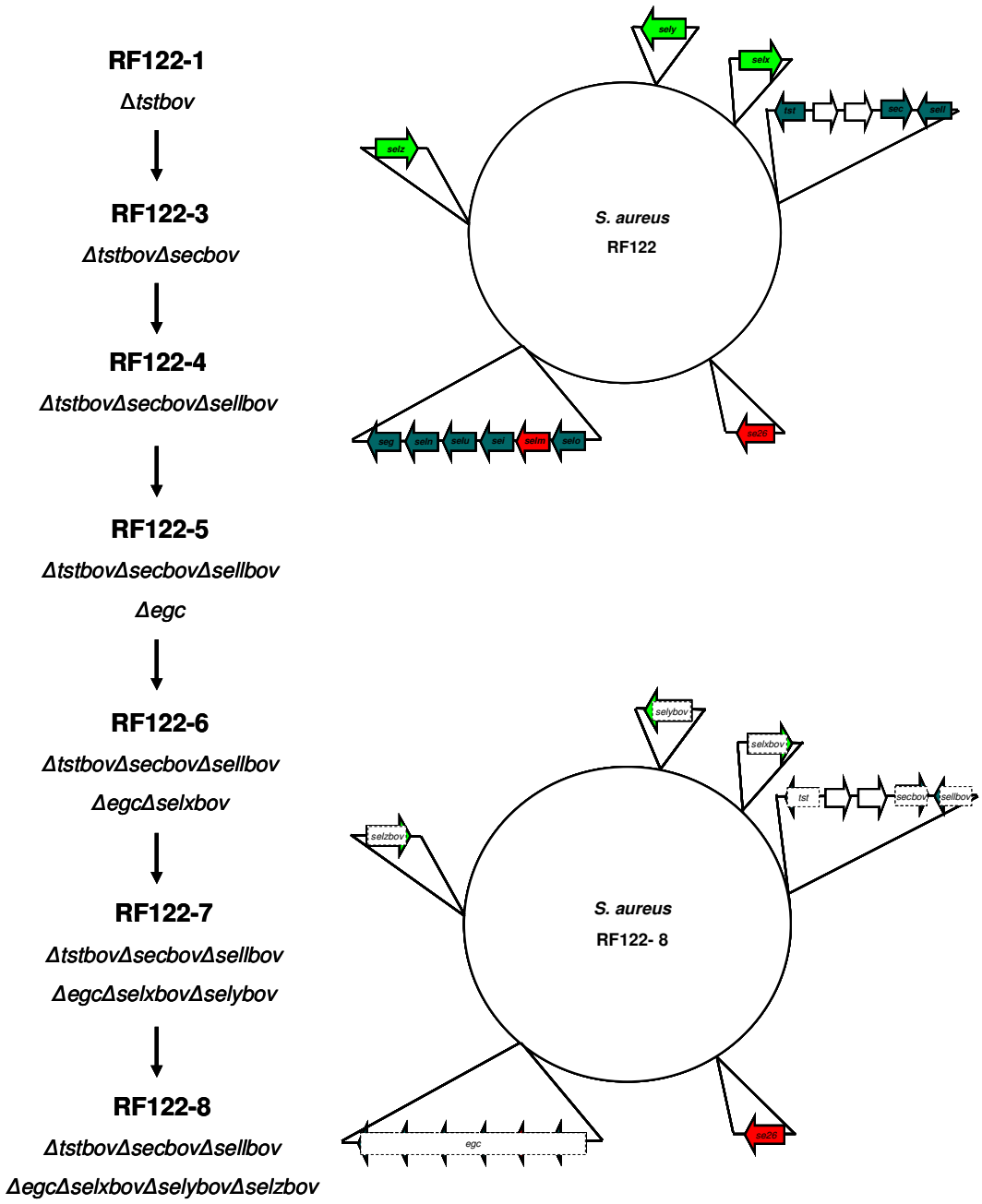


Figure 6.5: Schematic of sequential allele replacement events in *S. aureus* RF122-1 resulting in the generation of RF122-8. Dark green arrows indicate bovine variants of characterised SAg genes, light green arrows denote putative SAg genes, and red arrows represent SAg pseudogenes. Unfilled arrows indicate genes encoding pathogenicity island proteins. Boxes with broken lines indicate deleted regions.

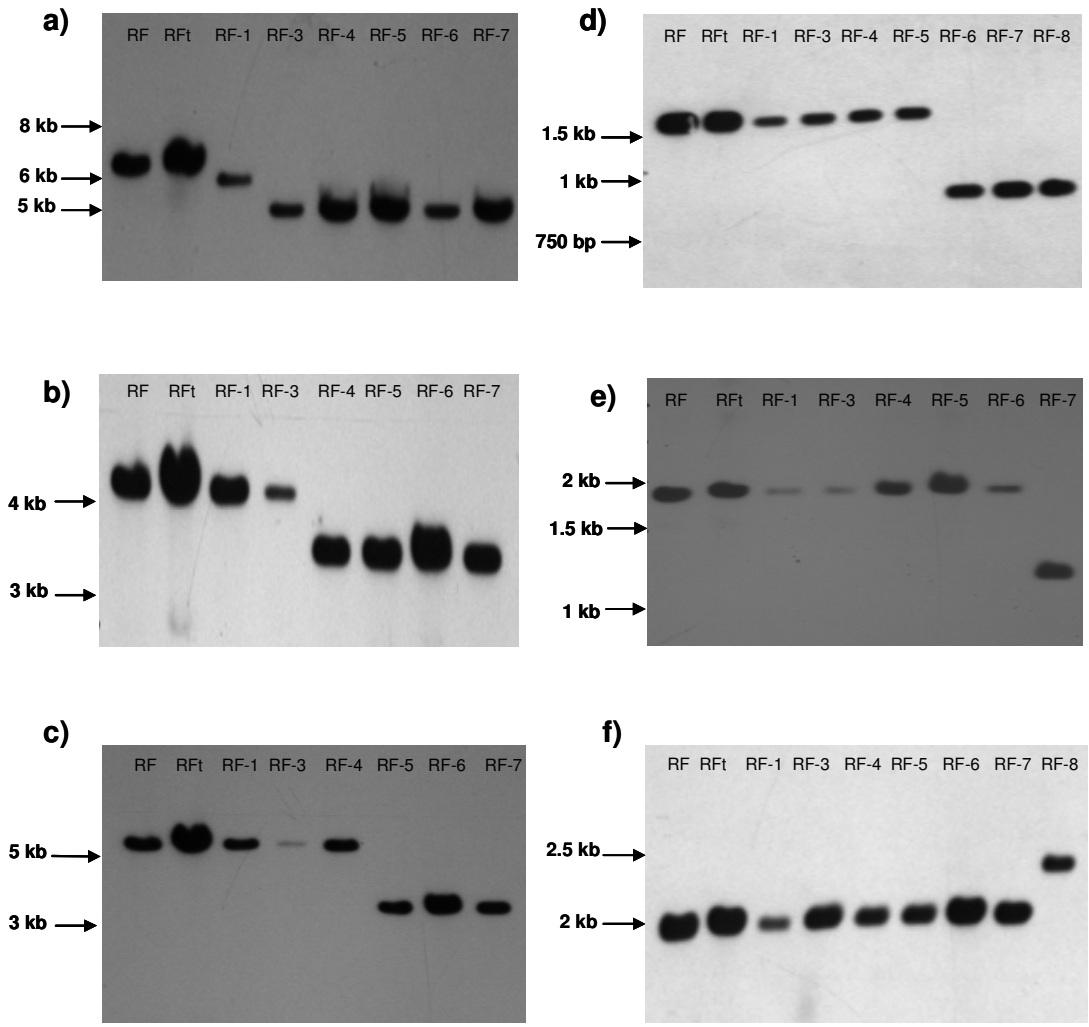


Figure 6.6: Southern blot analysis to confirm deletion of SAg genes. a) *secbov* **b)** *sellbov* **c)** *egc* **d)** *selxbov* **e)** *selybov* **f)** *selzbov* hybridised with gene-specific probes. Lanes 1-8 contain *S. aureus* strains RF122, RF122t, RF122-1, RF122-3, RF122-4, RF122-5, RF122-6, RF122-7 and RF122-8, respectively.

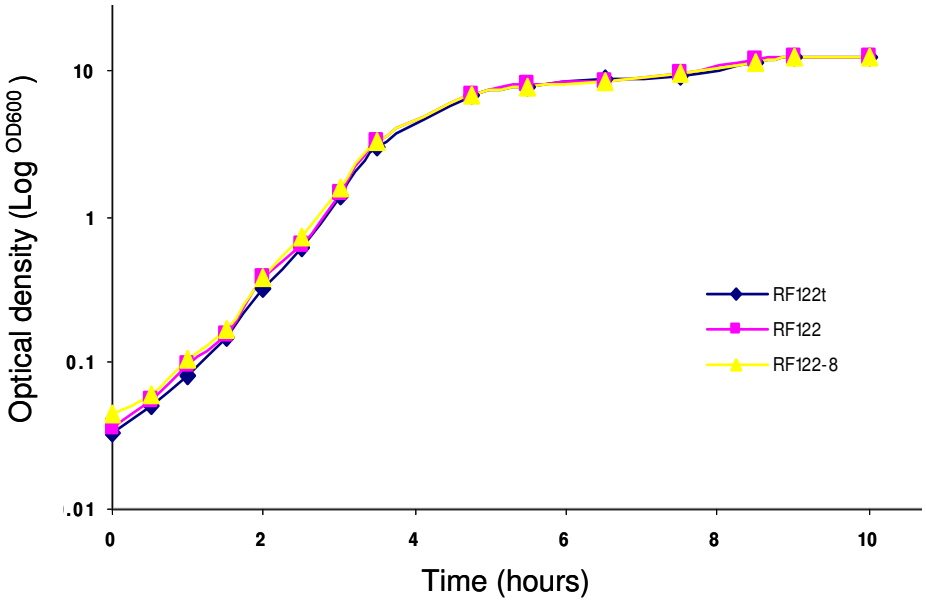
mutant strain was the expected size in relation to the wild type, indicating that the predicted deletion event had taken place (Figure 6.6).

6.3.2 Phenotypic analysis of the RF122 and SAg-deficient derivative RF122-8

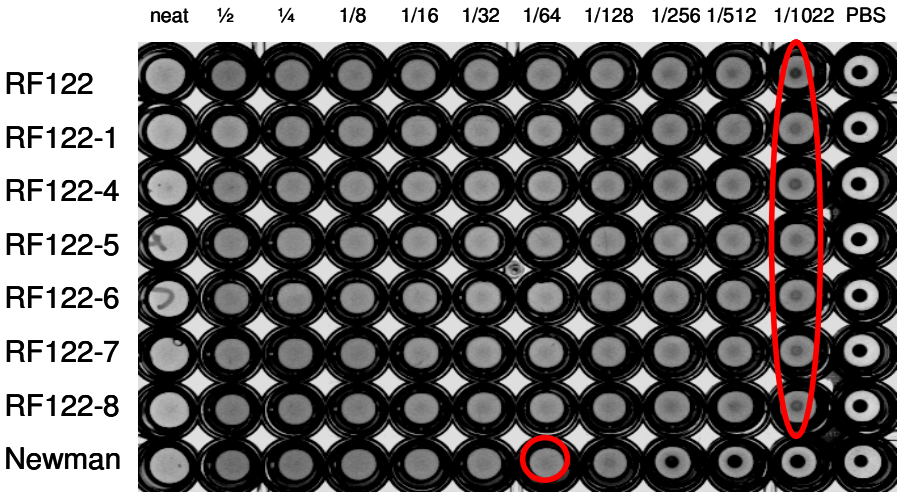
To investigate the possibility that deletion of SAg genes could have pleiotropic effects, the phenotype of WT and mutant strains was compared. It was also important to rule out the possibility that any undesired mutations had occurred during passage, such as in the *agr* locus (Somerville *et al.*, 2002). Firstly, a growth curve was determined for RF122, RF122t and RF122-8 grown in BHI liquid culture for 10 h at 37 °C, which revealed growth rates and yields were similar for each strain (Figure 6.7). In addition, the haemolysis of rabbit erythrocytes incubated with culture supernatants of RF122 and SAg-deficient derivative strains was investigated. In each case the haemolytic titre of RF122 and SAg-deficient derivatives was 1022, indicating that the deletion of SAg genes had no effect on haemolytic activity and that the *agr* locus was functional (Figure 6.7). Furthermore, analysis of the profile of secreted and CWA proteins of WT and mutant strains revealed no unexpected differences (Figure 6.7). The only observed difference is the expected absence of SEC_{bov}, a secreted protein of approximately 26 kDa, in strains RF122-3, RF122-4, RF122-5, RF122-6, RF122-7 and RF122-8, indicated by a red arrow in Figure 6.7.

Stationary and mid-exponential phase culture supernatants (10 ng/ml) of RF122 and RF122-8 were used to stimulate CFSE stained bovine PBMC to investigate their mitogenic activity. RF122-8 does not induce proliferation of bovine T-cells in either phase of growth, confirming loss of mitogenicity due to the deletion of all SAgs (Figure 6.8). RF122 induces higher level of T-cell proliferation in stationary than mid-exponential phase, which is consistent with the high level of transcription of SAg genes in stationary phase observed in this project. The level of T-cell proliferation of CFSE stained cultures in response to stimulation with RF122 appears to be lower than was measured by thymidine incorporation (Figure 6.11), which may be related to the efficiency of the staining method.

a)


Optical density (Log OD600)
10
1
0.1
.01
0
2
4
6
8
10
Time (hours)
RF122t
RF122
RF122-8

b)


neat
½
¼
1/8
1/16
1/32
1/64
1/128
1/256
1/512
1/1022
PBS
RF122
RF122-1
RF122-4
RF122-5
RF122-6
RF122-7
RF122-8
Newman

c)

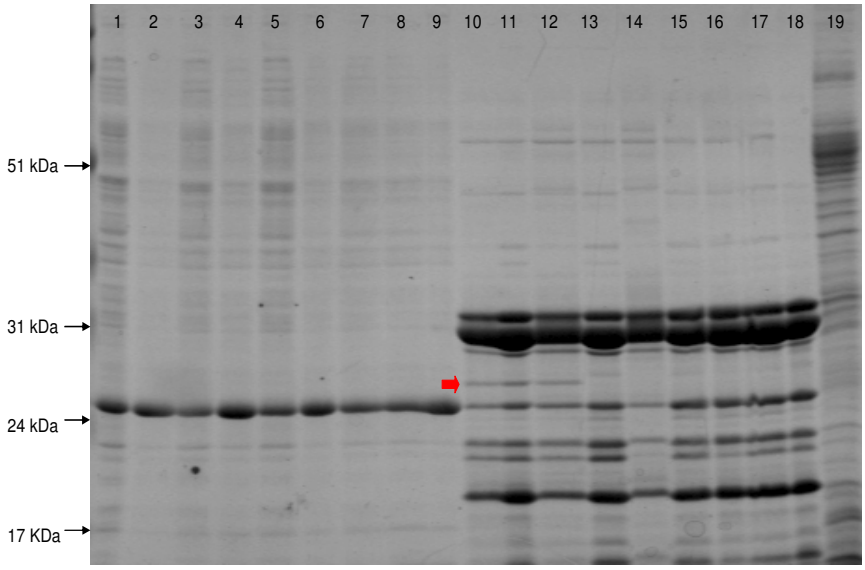

cell wall
secreted
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
51 kDa
31 kDa
24 kDa
17 KDa

Figure 6.7: Phenotypic analysis of the RF122 and SAg-deficient derivative RF122-8 a) Growth curve of *S. aureus* strains RF122t, RF122 and RF122-8 grown in BHI at 37 °C. **b)** Haemolytic activity of *S. aureus* strains Newman, RF122 and mutant strains. Experiments were performed at least in triplicate. End points of haemolysis have been circled in red. **c)** SDS-PAGE analysis of secreted and CWA proteins extracted from stationary phase cultures of *S. aureus.* SEC_{bov} protein is indicated by a red arrow. Lane 1, RF122; lane 2, RF122t; lane 3, RF122-1; lane 4, RF122-3; lane 5, RF122-4; lane 6, RF122-5; lane 7, RF122-6; lane 8, RF122-7; lane 9, RF122-8; lane 10, RF122; lane 11, RF122t; lane 12, RF122-1; lane 13, RF122-3; lane 14, RF122-4; lane 15, RF122- 5; lane16, RF122-6; lane 17, RF122-7; lane 18, RF122-8; lane 19, RN4220.

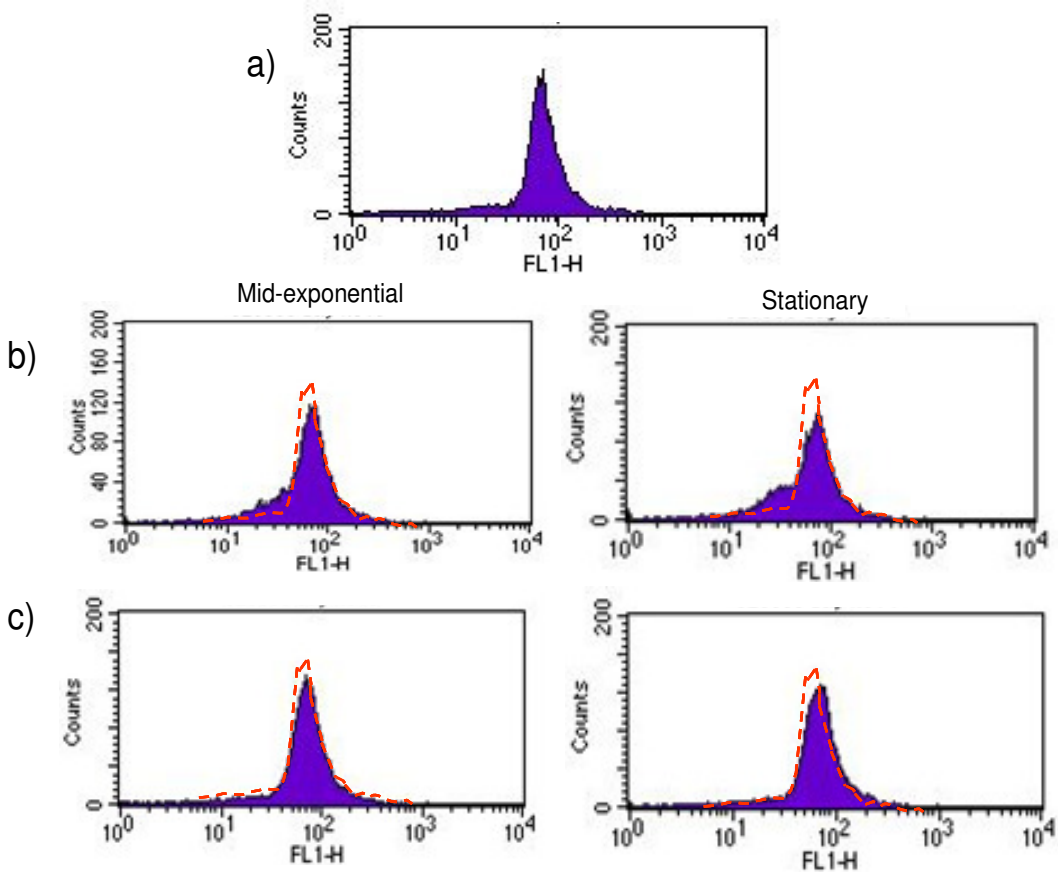


Figure 6.8: FACS analysis of CFSE stained bovine PBLs after stimulation with a) medium only, b) RF122 and c) RF122-8 stationary and mid-exponential phase culture supernatants for 4 d. Red broken lines indicate the data obtained for stimulation with medium only.

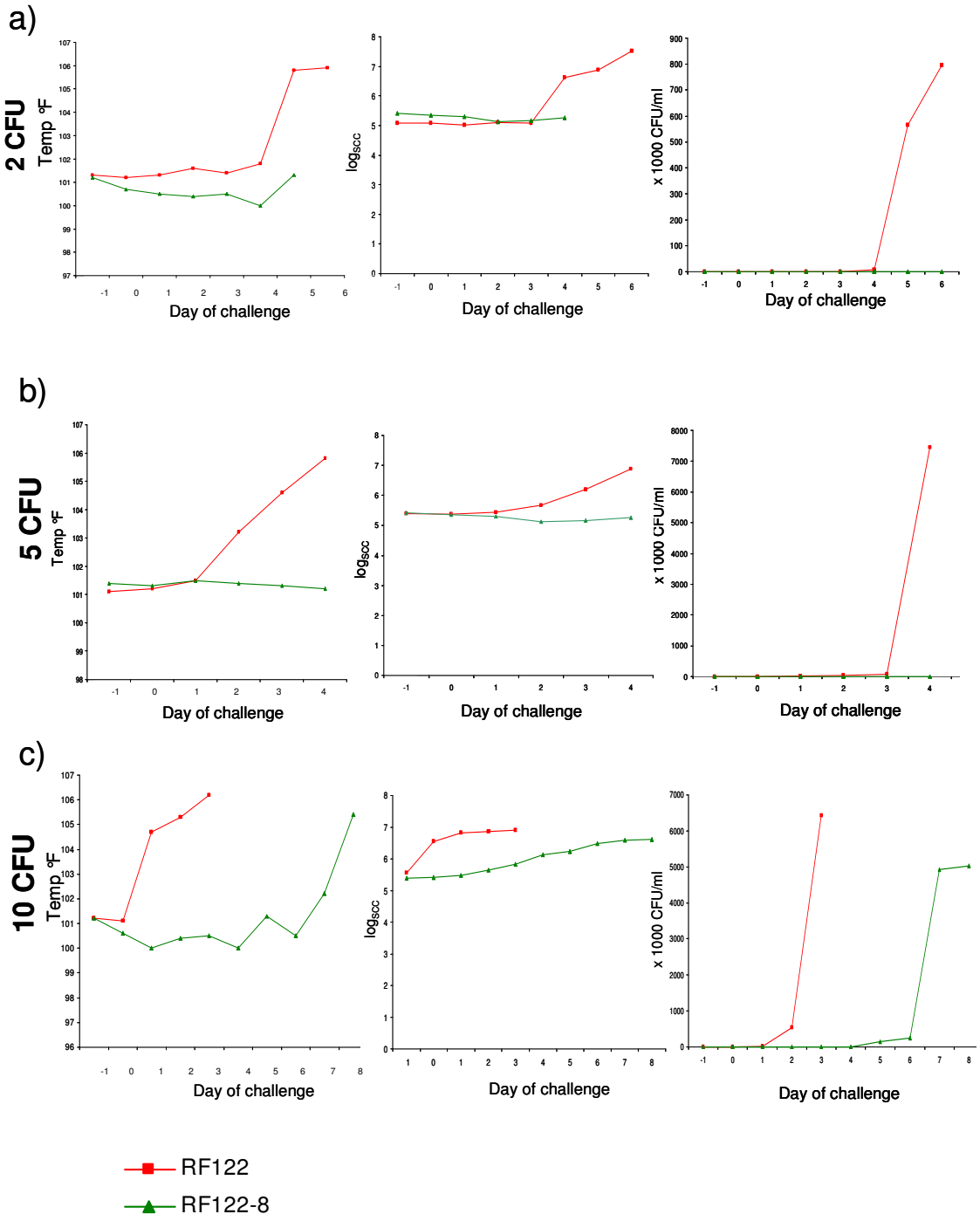


Figure 6.9: **Experimental IMI of dairy cattle**. Infectious dose of RF122 and RF122-8 was determined by instillation of **a)** 2 CFU, **b)** 5 CFU and **c)** 10 CFU of RF122 and RF122-8 directly into the udder of each cow. Body temperature, SCC, and bacterial counts obtained from the animals were measured daily throughout the course of infection.

6.3.3 SAg-deficient strain RF122-8 requires a higher infectious dose than wild type RF122 to establish experimental infection of dairy cattle

Preliminary experiments were carried out to determine the infectious dose of each strain, for IMI of cows. An initial inoculum of 2 CFU of either RF122 or RF122-8 was introduced directly into the udder. Infection was established with RF122 after 3 d, however RF122-8 was not infectious at this dose (Figure 6.9). Similarly, instillation of 5 CFU resulted in an infection with RF122 after 3 d, but not with RF122-8 (Figure 6.9). Symptoms of acute clinical mastitis were observed during infection including swelling of the udder, high body temperature and high SCC which indicates a strong inflammatory response caused by bacterial infection. Elevation of body temperature above 41 °C (105 °F) indicated a high risk for loss of the animals (University of Idaho animal use guidelines). Therefore experiments were stopped by treatment with anti-inflammatory drug (Benamin), aspirin, and penicilin G. Establishment of infection with strain RF122-8 required an inoculum of 10 CFU, and once established the clinical symptoms associated with each strain were similar including high SCC of 6 Log^{SCC} and elevation of body temperature above 41 °C (105 °F) (Figure 6.9). These studies have shown that despite evidence of some attenuation of the SAg-deficient derivative of RF122 reflected in differing infectious doses, both strains are infectious at extremely low doses and are highly virulent in their natural host.

6.3.4 Construction of α-toxin-deficient derivatives of RF122 and RF122-8

The University of Idaho guidelines associated with large animal experimentation require animals to be treated with antibiotics when there is high risk of death, which has proven problematic for studying pathogenesis of highly virulent strains RF122 and RF122-8. To allow the experimental infections to be carried out over the full course of infection, it is necessary to attempt to reduce the virulence of the strains. In this study we have demonstrated that the culture supernatant of RF122 exhibits a high level of haemolytic activity when incubated with rabbit erythrocytes (Figure 6.10), due at least in part to high levels of α-toxin production. Animal infection studies have shown that strains containing the toxin are more virulent than their isogenic derivatives in a murine model of mastitis (Bramley *et al*., 1989). Deletion

of *hla* was carried out in strains RF122 and RF122-8 with the aim to reduce virulence and allow the effect of both *hla* and SAg gene deletions in mastitis to be elucidated. pMAD:*hla* was constructed by cloning the spliced fragments flanking the *hla* gene between the *Sal*I and *Sma*I sites of pMAD. Digestion of pMAD:*hla* with *Eco*RI confirmed the expected restriction pattern of the insert, 814 bp and 428 bp (Figure 6.3). pMAD:*hla* was transformed into RF122 and RF122-8 and *hla*-deficient derivatives were obtained by allelic replacement. The region spanning the *hla* gene amplified from strains RF122t and RF122-8 is 2229 bp, and 1241 bp in RF122t-α and RF122-8α, indicating a deletion of 988 bp from the *hla* gene (Figure 6.4)**.** In addition, sequencing across the region in RF122t-α and RF122-8α was carried out to verify an in frame deletion has occurred at the appropriate site.

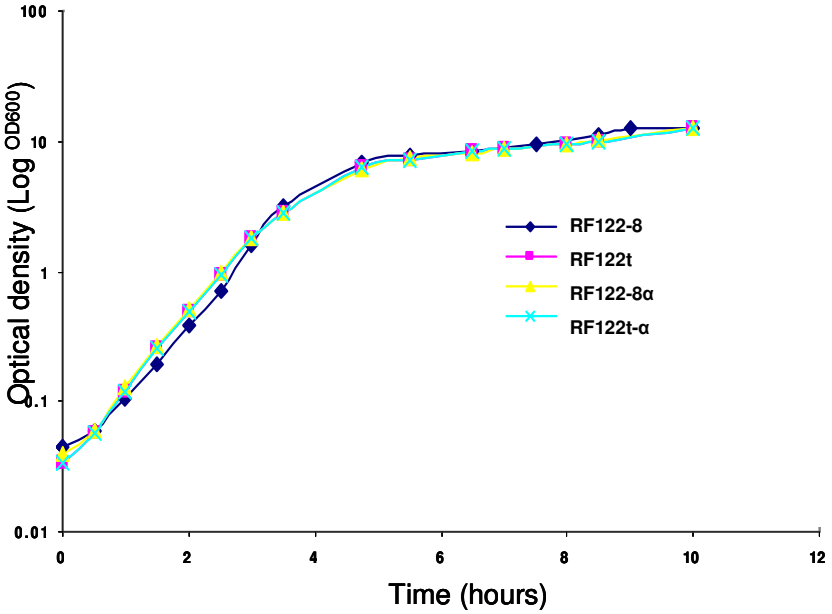
6.3.5 Phenotypic verification of α toxin deficient derivatives of RF122 and RF122-8

Phenotypic tests were carried out to investigate the effect of the *hla* deletion on the strains RF122t-α and RF122-8α. Firstly, a growth curve was determined for RF122, RF122t, RF122t-α, RF122-8 and RF122-8α grown in BHI liquid culture for 10 h at 37 °C, which revealed growth rates are unchanged by deletion of the *hla* gene (Figure 6.10). Similarly analysis of the secreted and CWA protein profiles produced by strains RF122t, RF122t-α, RF122-8 and RF122-8α by SDS-PAGE revealed no unexpected changes (Figure 6.10). The 33 kDa α-toxin could not be visualised on the SDS-PAGE gel, but is thought to be masked by a highly abundant 31 kDa protein.

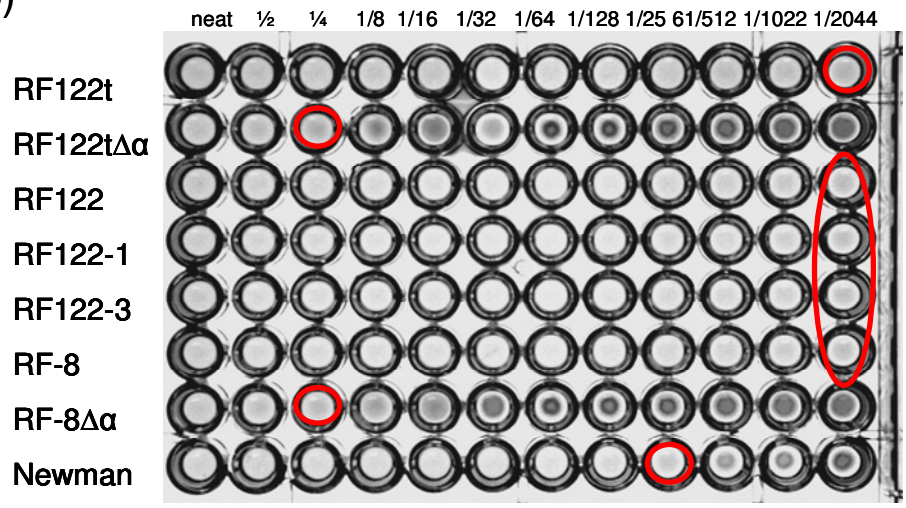
Stationary phase culture supernatants of RF122t, RF122t-α, RF122-8 and RF122-8α were used to stimulate bovine PBMC to investigate their mitogenic activity. As expected, RF122-8 and RF122-8α do not induce proliferation of bovine T-cells confirming loss of mitogenicity due to the deletion of all SAgs in both strains (Figure 6.10). RF122t and RF122t-α induce high levels of T-cell proliferation, however it is noteworthy that there is a slight decrease in proliferation associated with RF122t-α. The haemolysis of rabbit erythrocytes incubated with culture supernatants was investigated and confirmed a drastically reduced haemolytic titre of 4 associated with *hla*-deficient strains RF122t-α and RF122-8α, compared to a haemolytic titre of 2044 for WT and SAg-deficient derivatives

(Figure 6.10). Experimental infection of dairy cattle with RF122t-α and RF122-8α will be carried out in early 2011.

a)


100
10
1
0.1
0.01
Optical density (Log OD600)
0
2
4
6
8
10
12
Time (hours)
RF122-8
RF122t
RF122-8α
RF122t-α

b)


neat
½
¼
1/8
1/16
1/32
1/64
1/128
1/25
61/512
1/1022
1/2044
RF122t
RF122tΔα
RF122
RF122-1
RF122-3
RF-8
RF-8Δα
Newman

c)

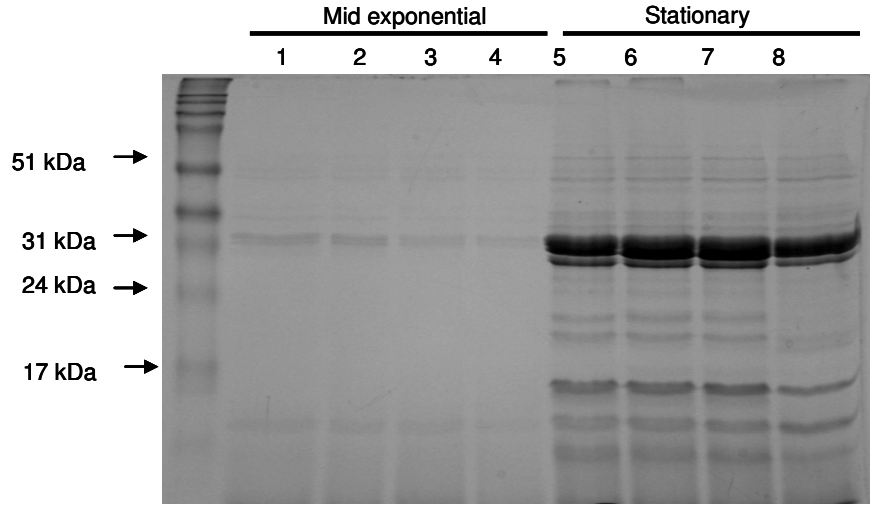

Mid exponential
Stationary
1
2
3
4
5
6
7
8
51 kDa
31 kDa
24 kDa
17 kDa

Figure 6.10: Phenotypic verification of α toxin-deficient derivatives of RF122 and RF122-8. a) Growth curve of *S. aureus* strains RF122t, RF122t-α, RF122-8 and RF122-8α grown in BHI at 37 °C. **b)** Haemolytic activity of *S. aureus* RF122 and mutant strains. End points of haemolysis have been circled in red. **c)** Coomassie stained SDS-PAGE analysis of and stationary phase. Lanes 1 to 4 contain secreted proteins from exponential phase cultures of RF122t, RF122tΔα, RF122-8 and RF122-8 Δα, Lanes 5 to 8, secreted proteins from stationary phase cultures of RF122t, RF122tΔα, RF122-8 and RF122-8 Δα.

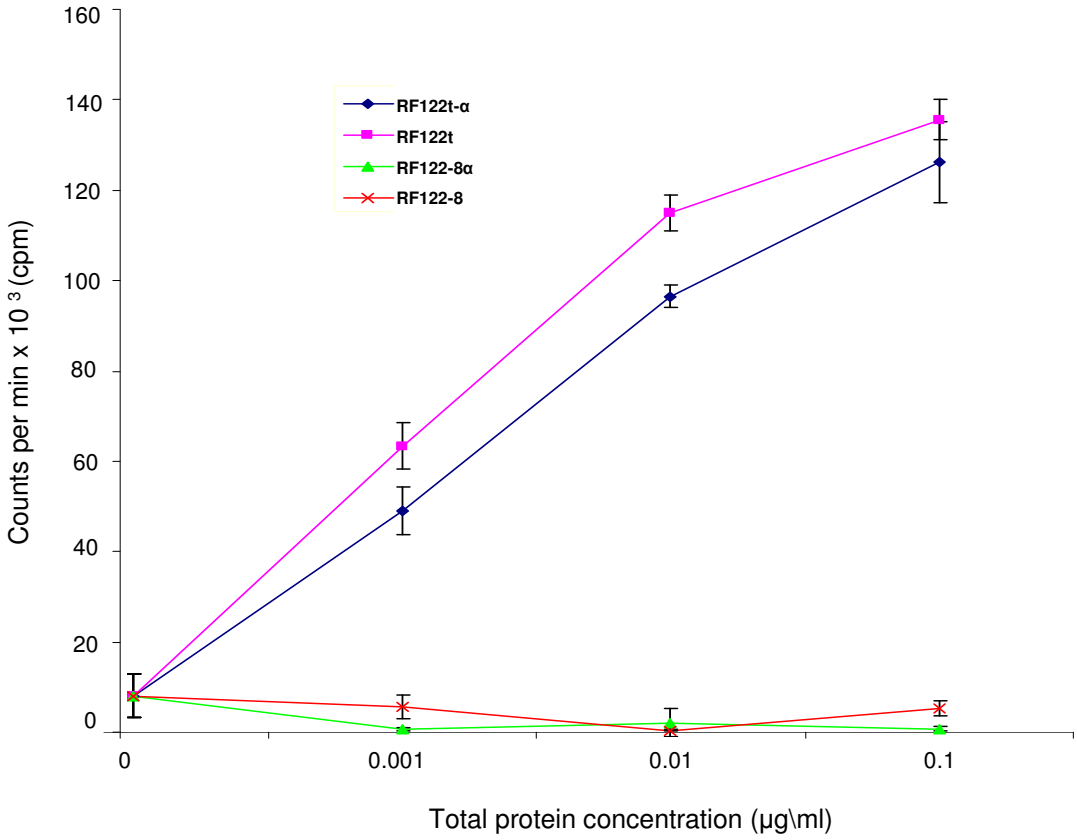


Figure 6.11: Bovine T-cell activation by RF122t, RF122t-α, RF122-8 and RF122-8α. PBMC proliferation after 4 d exposure to *S. aureus* supernatants, as indicated by the incorporation of [^{3}H] thymidine. Experiments were performed in triplicate and error bars were included to represent SD.

6.4 Discussion

A small number of pathogenic clones of *S. aureus* are responsible for the majority of bovine mastitis infections (Smyth *et al.*, 2009). In this study, analysis of the genome of *S. aureus* RF122, a representative isolate of the common bovine clone ST151, revealed the full complement of SAg genes encoded by this strain. Targeted disruption of staphylococcal genes leading to loss of function is an important tool in the study of *S. aureus* disease pathogenesis, allowing the contribution of individual genes to be determined. Antibiotic resistance cassettes are often used as markers for mutated alleles however this limits the number of genes that can be inactivated in a single strain. Gene disruption using the pMAD plasmid overcomes this limitation by the incorporation of a constitutively expressed β-galactosidase gene to allow blue/white colony screening for double crossover events (Arnaud *et al*., 2004).

SaPIbov was originally characterised in RF122, and *tstbov*-deficient RF122-1 and *tstbov* and *secbov*-deficient RF122-2 isolates were obtained by disruption with tetracycline and erythromycin resistance cassettes respectively (Fitzgerald *et al*., 2001a). In the study by Fitzgerald *et al*, residual SAg activity was observed in the absence of *secbov* and *tstbov*, which indicated that RF122 expressed additional SAgs. This is consistent with our finding that RF122 has the potential to express a further 9 SAgs. In the current study we have deleted all remaining SAg genes in RF122-1 by sequential allele replacement with pMAD gene deletion constructs, leaving no additional antibiotic resistance markers. Many strains of *S. aureus* are not transformable and require transformation into an intermediate restriction-modification negative host such as RN4220 before phage transduction into the strain of interest (Kreiswirth *et al.*, 1983). In this study we have been able to introduce deletion plasmids directly into the strains of interest. The reason for the unusually high transformation efficiency of RF122t and derivative strains is currently unknown, but could be the result of a mutation in a restriction-modification pathway which has occurred during passage.

The resulting strain RF122-8 is deficient in SAg genes, and represents a valuable tool which can be used in wide range of both *in vitro* and *in vivo* studies. For example, we have described expression of individual SAg genes in this strain,

which has allowed Vβ specific lymphocyte expansion in response to specific SAg proteins to be investigated. The deletion of all SAgs in RF122-8 had the expected effect of abrogating mitogenicity of culture supernatants. Importantly, comparable levels of haemolysis and similar protein profiles obtained for WT and mutant strains suggests there have been no unexpected mutations affecting *agr* function during passage of the strains. Previously, *in vitro* passage of *S. aureus* has resulted in mutations within the *agr* operon (Somerville *et al.*, 2002). Vojtov *et al*, reported the ability of some SAgs to act as global repressors of exotoxin synthesis, where SDS-PAGE analysis revealed a significant reduction in levels of exoprotein production when *tst* was inactivated (Vojtov *et al.*, 2002a). In contrast, we have demonstrated that the deletion of all SAg genes in RF122, including *tstbov* had no effect on protein synthesis. *tstbov* is an allelic variant of *tst* with 98% nucleotide identity, which may explain the lack of exoprotein repression associated with TSST-1_{bov}, alternatively it could be due to the RF122 strain background.

SAgs play a direct role in food poisoning and toxic shock syndrome (TSS) however their role in other diseases such as mastitis is less well understood. In this study SAg-deficient strain RF122-8 was constructed to determine the effect of SAgs produced during staphylococcal IMI on cytokine release, leukocyte sub-population changes, progression of disease severity and persistence. The infectious dose of each strain was determined by direct instillation of bacteria into the udder of the cows. Remarkably, infection was established with only 2 CFU of RF122, indicating the highly virulent nature of the strain.

RF122-8 required a higher inoculum of 10 CFU to establish infection. However once infection was established the clinical symptoms associated with each strain were similar and typical of acute clinical mastitis including; swelling of the udder, high SCC (6 Log^{SCC}) and elevation of body temperature above 41 °C (105 °F). These studies have shown that despite an evident attenuation in infectious dose of the SAg-deficient derivative of RF122, both strains are infectious at very low doses and are highly virulent in their natural host. Previous experimental intra-mammary infection studies have described infectious doses ranging from as low as 50 CFU for strains M60 and 409 (Tollersrud *et al.*, 2006) to 10^3 CFU for strain RC108 and 10^4 CFU of strains 510, 571, and 581 (Pellegrino *et al.*, 2008). 50 CFU of strain 409 was an optimal dose to establish a subclinical mastitis model

(Tollersrud *et al.,* 2006). It is remarkable that an infectious dose of only 2 CFU of RF122 causes symptoms of acute clinical mastitis. In contrast, an infectious dose of 10^8 CFU is necessary to establish infection of mice with ST151 isolates which are clonally related to RF122 (Guinane *et al*., 2008). This is consistent with the intrinsic sensitivity of natural hosts when exposed to host-specialised *S. aureus* strains. The mouse model is a useful tool for studying mastitis but such large variations in infectivity highlight the importance of studying the pathogenesis of bacterial strains in their natural host.

The progression of disease during staphylococcal mastitis often begins with an acute clinical episode as observed during infection with RF122 and RF122-8, which then develops into a persistent chronic infection (Anderson, 1982). Unfortunately due to high risk of animal loss, experiments were stopped prematurely and we were unable to monitor the animals throughout the full course of intra-mammary infection (IMI). The guidelines associated with large animal experimentation which require animals to be treated with antibiotics when there is high risk of death, have proven problematic for studying pathogenesis of these strains. To allow the experimental infections to be carried out over the full course of infection, it is necessary to reduce the virulence of the wild type and SAg-deficient strains. Analysis of the genome sequence of RF122 revealed that in addition to SAgs, RF122 has the potential to produce many toxins which could potentially be responsible for the increased virulence of the strain, including SSLs, α- and β-toxin, exfoliative toxins, streptolysin homologs, leukocidins and leukotoxins (Herron-Olson *et al*., 2007). α-haemolysin is a pore forming toxin which promotes cell lysis and release of internalised bacteria, and is produced by almost all bovine isolates of *S. aureus* (Kenny *et al.*, 1992). Animal infection studies have shown that strains producing the toxin are more virulent than their isogenic derivatives in a murine model of mastitis (Bramley *et al*., 1989). Previously, it has been demonstrated that strain RF122 produces high levels of α-toxin (Guinane, 2008). Therefore we have constructed α-toxin-deficient derivatives of RF122 and RF122-8 to allow future experiments to be carried out to determine the role of SAgs produced during staphylococcal IMI. RF122t and *hla*-deficient RF122t-α induce high levels of T-cell proliferation, however it is noteworthy that we have observed a slight decrease in proliferation associated with RF122t-α which could be due to a potential

synergestic mode of action of SAg and α-toxin. α-toxin has recently been shown to augment penetratation of TSST-1 through porcine vaginal tissue in an *ex vivo* model (Brosnahan *et al*., 2009), but it is unknown whether a similar phenomenon is observed in the bovine udder. Future experiments utilizing the *hla* and SAg-deficient strains constructed in this study would allow investigation into this hypothesis.

In this study, preliminary experimental IMI has been carried out and revealed attenuation of the infectious dose of a SAg-deficient derivative of RF122 compared with WT. We have also constructed *hla*-deficient derivatives of RF122 and RF122-8 which will be used in ongoing experimental infection of dairy cattle.

Chapter 7
General discussion

SAgs are known to play a direct role in the pathogenesis of diseases such as food poisoning and TSS (Dinges et al., 2000), but SAg genes are harboured by strains isolated from a wide range of hosts and disease manifestations (Holtfreter *et al*., 2007). Although SAgs are known to contribute to immunomodulation, the consequences of this activity for disease progression are not well understood. In particular, the role of SAgs in bovine mastitis has not been investigated in detail. In recent years, there has been an exponential increase in the number of *S. aureus* genome sequences submitted to public databases such as NCBI. This information has been valuable in the identification of genes encoding novel virulence factors. Most of the sequenced strains are of human origin, but genomes of isolates from bovine, ovine and avian hosts have recently been included, facilitating comparative genomic analysis of staphylococcal adaptation to different host species (Guinane *et al*., Lowder *et al*., 2009, Herron-Olson *et al*., 2007). In the current study, the genome sequence for bovine strain RF122 allowed us to perform a genome scale analysis of the presence of characterised or novel SAg genes.

Previously, SaPIbov which contains genes encoding SEC_{bov}, $SElL_{bov}$ and $TSST\text{-}1_{bov}$ was discovered in strain RF122 (Fitzgerald *et al*., 2001a). In addition, we have identified bovine variants of characterised SAgs encoded by the *egc* including SEG, SEI, SElO, SElN and SElU, and 3 novel putative SAg genes. The novel SAgs genes were found in a number of human and ovine lineages, in addition to bovine strains. It is likely that as additional genome sequences are released, more SAgs will be discovered. *S. aureus* is now known to encode 25 distinct SAgs, SEA through to E, SEG to J, SER to SET, SElK to Q, SElU, SElV, and SElX to SElZ and TSST-1. In addition there are a further 2 putative SAg genes *selw* and *se26* which have not been characterised.

Regulation of a number of SAgs including SEC and TSST-1 has been shown to be *agr* dependent (Novick, 2003). We investigated the transcription of all RF122-encoded SAg genes and found that *secbov*, *sellbov*, *tstbov* and *selxbov* are upregulated in stationary phase, consistent with regulatory control by *agr*. Of note, ST151 strains were previously demonstrated to have higher levels of RNAIII transcription in comparison with other ruminant clones (Guinane *et al*., 2008). Further, although ST151 strains contain an Agr group II operon, AgrC is encoded by a novel allele and *sarU* and *sarT* genes are absent (Herron-Olson *et al*., 2007).

There is also a novel transcriptional regulator within a gene cluster previously shown to affect *agr* expression (Shaw *et al.*, 2006) which may contribute to the elevated RNAIII levels (Guinane *et al*., 2008). Taken together, these data suggest that ST151 strains have a unique *agr* expression phenotype which may influence SAg production. We have also observed upregulation of some SAgs including $SElY_{bov}$ and $SElZ_{bov}$ in mid-exponential phase. The distinct regulation among SAg genes encoded by strain RF122 implies specific SAgs may be expressed at different stages of infection.

Our observation that *egc* genes are transcribed at very low levels in both mid-exponential and stationary phase of growth, is consistent with Derzelle *et al* who reported low *egc* transcript levels among 28 human strains (Derzelle *et al.*, 2009). It is possible that the *egc* genes may be expressed at higher levels *in vivo*, as has been observed for the streptococcal SAgs SPEA and SPEC (Kazmi *et al*., 2001, Broudy *et al*., 2001). SPEA was produced in small quantities *in vitro,* but in the BALB/c mouse, SPEA production was significantly increased (Kazmi *et al*., 2001), and SPEC production has been shown to increase in co-culture with human pharyngeal cells (Broudy *et al*., 2001). However, despite the relatively high prevalence of the *egc* cluster among clinical isolates of *S. aureus*, neutralising antibodies are rare (Holtfreter *et al*., 2004). It has been demonstrated that less than 10% of normal human serum samples contain neutralising antibody specific for *egc* SAgs, in comparison with between 32% and 86% of individuals which have antibody against selected non-*egc* encoded SAgs (Holtfreter *et al*., 2004). Similarly, in this study we have observed lower levels of antibody against *egc*-encoded SAgs, SEG_{bov}, $SElO_{bov}$, $SElU_{bov}$ and SEI_{bov} in comparison with the novel SAgs, $SElX_{bov}$, $SElY_{bov}$ and $SElZ_{bov}$, in sera obtained from humans and animals. The low transcription levels of *egc* genes, taken together with the low levels of antibodies detected, leads us to speculate that the *egc*-encoded SAgs may be less important than other SAgs in *S. aureus* disease pathogenesis, including bovine mastitis. Furthermore in the RF122 genome, *selmbov* and *segbov* are predicted to encode truncated proteins, which may not be functional, consistent with a limited role for the *egc* locus in the pathogenesis of bovine pathogenesis.

Prior to this study all identified SAgs produced by *S. aureus* were known to be encoded by MGEs or highly variable chromosomal regions (Fitzgerald et al.,

2001a, Jarraud *et al*., 2001, Johns & Khan, 1988, Ono *et al*., 2008, Ben Zakour *et* al., 2008). Here we have identified and characterised SElX, which is the first SAg encoded by the core genome of *S. aureus* and found in the great majority of strains. SElX is most closely related to TSST-1 and belongs to phylogenetic group IV of staphylococcal SAgs. Fraser *et al* proposed that TSST-1 was the sole survivor of an early divergence between the related toxin families, SAgs and SSLs (Fraser & Proft, 2008). TSST-1 is phylogenetically most closely related to the SSLs, but has the biological function of a SAg. It is likely that SElX is a second example of an intermediate survivor between these two groups. Structural modelling of SElX revealed it has a unique predicted structure with a much smaller B domain than that of other SAgs identified to date and the OB fold is absent. The well conserved and successful 2-domain structure common to SAgs and SSLs, is thought to have evolved through recombination of 2 small ancestral β-strand motifs, predicted to be Ig-binding motifs of streptococcal proteins G and L, and the OB fold of the heat labile enterotoxin family which resemble domains A and B respectively, where conserved amino acids in the central alpha helix brought 2 domains together (Mitchell *et al*., 2000). Previously, it was shown that *Bordetella pertussis* cytolysin, which has both adenylate cyclase and haemolytic activity evolved through a similar mechanism (Glaser *et al.*, 1988). SElX therefore represents an intermediate that lacks the OB fold, which could have important implications for our understanding of the structural evolution of the SAg family. Ongoing crystallography analyses should result in important insights into the molecular interaction of SElX with its ligands which may facilitate the design of molecules to inhibit its function. The predicted structure of SElX is most closely related to TSST-1 and SSL7. SSL7 has two functional sites, the OB-fold which binds to the FcαR1 portion of the IgA Fc region is missing in SElX, however the C-terminal A domain which binds to the C5 component of complement is similar (Fraser & Proft, 2008). It would therefore be important to investigate the possibility that SElX is a hybrid molecule which has both SSL and SAg activity by investigating its capacity to inhibit complement.

In this study we have demonstrated that bovine- and ovine-specific SElX variants have increased mitogenicity and a distinct bovVβ T-cell activation profile in comparison with a human SElX variant suggesting that SElX has undergone adaptive diversification leading to enhanced activity in ruminants. This indicates

that *S. aureus* is able to produce highly adapted SAg variants with optimal efficiency in their natural hosts, but which are functional across a diverse range of hosts. We have identified 11 distinct *selx* alleles from divergent human lineages, and further investigation of human-specific allelic variants may reveal similar functional diversification or antigenic variation. Importantly, we have described high levels of expression of SElX by the CA-MRSA strain USA300 LAC, which may contribute to the characteristic enhanced virulence of this strain. Ongoing experiments with LAC and an isogenic SElX-deficient derivative in a rabbit model of necrotising pneumonia will allow us to determine the importance of this novel SAg in virulence.

For the first time in this study we have comprehensively evaluated bovVβ-specific T-cell activation in response to stimulation with all SAgs encoded by a single successful mastitis isolate. We have established that RF122 has the potential to stimulate the entire bovVβ repertoire by expression of SAgs. It is possible that this capacity allows the bacteria to contend with host genetic variation in the expressed TCR Vβ repertoire among the cattle population. It is also feasible that SAgs with distinct bovVβ activation profiles are expressed at different stages of infection, potentially indicating a role for SAgs in persistence. In future experiments, it will be important to address whether the proportion of T-cells expressing Vβ genes varies between animals, and the effect particular frequencies have on susceptibility to mastitis caused by *S. aureus*. In addition, it will be important to examine the Vβ specificity of T cells activated during future experimental mammary gland infections with *S. aureus*.

To determine the role of SAgs in bovine mastitis, we have constructed a SAg-deficient derivative of RF122 by sequential allele replacement, which provides a unique tool to investigate SAg function in *in vitro* and *in vivo* studies. Previous studies have monitored the effects of intra-mammary instillation of purified SEC1 or a laboratory strain RN4220 with a plasmid containing the *sec1* gene (Ebling *et al.*, 2001, Kuroishi *et al.*, 2003). Kuroishi *et al* described migration of neutrophils into milk within 24 h after intramammary instillation of SEC1 (Kuroishi *et al.*, 2003). Ebling *et al* were unable to detect any alteration in the CD4 or CD8 T-cell populations in response to plasmid-encoded SEC1 during infection (Ebling *et al.*, 2001). However isogenic mutants of clinical isolates deficient in SAg production

have not been previously examined in experimental IMI. In this study we carried out experimental intra-mammary infection of dairy cattle with RF122 and the SAg-deficient strain RF122-8 with the aim to determine the effect of SAg expression during persistent infection. Unfortunately, we were unable to establish a subclinical mastitis infection, due to the hyper-virulence of strain RF122 in its natural host. ST151 strains are often isolated from subclinical infections (Fitzgerald *et al*., 1997, Fitzgerald *et al*., 2000), which suggests the route of delivery used in the current study may contribute to the hyper-virulence observed during experimental infection with RF122. It is feasible that damage to the teat canal at the injection site during direct intra-mammary inoculation may have contributed to the acute clinical outcome of infection. In addition, the bacteria used to inoculate the cows have been grown *in vitro* in TSB, a nutrient-rich growth medium which may lead to the expression of higher levels of toxins than would be associated with natural infection.

In spite of these limitations, our data suggest that SAgs play a role in early infection due to the observed attenuation of infectivity with the SAg-deficient mutant. RF122 was able to establish infection at a dose as low as 2 CFU, whereas RF122-8 required a dose of 10 CFU. Classically, SAgs have been implicated with modulation of the adaptive immune response, however there is mounting evidence that they also play a role in the early stages of infection. For example, SEC1 was shown to induce early migration of neutrophils to the mammary gland (Kuroishi *et al.*, 2003) and Dauwalder *et al* have also described early induction of leukocytes by SEA *in vitro* (Dauwalder *et al.*, 2009). Furthermore expression of SPEA2, SPEJ and SMEZ is upregulated during early infection of cynomolgus macacques in experimental pharyngitis (Virtaneva *et al*., 2005).

The extremely low inoculum required to establish infection of dairy cattle in this study is in stark contrast with the 10^8 CFU dose necessary to establish infection of mice with ST151 isolates (Guinane *et al*., 2008). To our knowledge, 2 CFU is by far the lowest inoculum that has been described to establish intra-mammary infection by bacteria. The mouse model is a useful tool for studying *S. aureus* pathogenesis but the large discrepancy in doseage required highlights the importance of studying the pathogenesis of bacterial strains in an appropriate animal host. This is particularly important when studying the effect of SAgs as the

murine and bovine immune T-cell repertoires are very different (Connelley *et al*., 2009, Bosc & Lefranc, 2000). In addition, it is well established that mice are much less sensitive to the effect of SAgs than humans due to a much lower binding affinity for murine than human MHC class II molecules (Sriskandan *et al*., 2001, DaSilva *et al.*, 2002, Yeung *et al.*, 1996, Faulkner *et al.*, 2005, Peavy *et al*., 1970).

In this study we have comprehensively evaluated bovine T-cell activation in response to SAg stimulation. Further experiments are necessary to characterise the interaction of SAgs with bovine MHC class II molecules, particularly as comparison between mastitis resistant and susceptible cows reveals an association with DH24A (DRB3.2*24 and DQA*1A, DQB*1 (Park *et al.*, 2004, Kelm *et al.*, 1997). Most SAgs including SEA, SEB, SED, SEH and TSST-1 are known to bind preferentially to human MHC class II HLA-DR molecules with the exception of SEC which binds preferentially to HLA-DQ (Herrmann, 1989). The cattle MHC is distinct from the human MHC in a number of ways including a chromosomal inversion, ruminant specific DYA and DYB genes (Ballingall *et al.*, 2004, Ellis, 2004), and a variable number of DQ genes. It would be interesting to investigate if the bovVβ profile is dependent on the HLA-DR allelic polymorphism or differences in SAg concentrations, as has been observed with the human Vβ response (Llewelyn *et al.*, 2006). Additionally, the bovine genome has recently been sequenced which leading to the identification of a large number of novel cytokines, attributed to extensive colonisation of the gut rumen (Elsik *et al*., 2009). Accordingly, further investigation into bovine cytokine release in response to SAgs is warranted.

SAgs are produced by a diverse range of microbial species. In addition to well characterised bacterial SAgs, a number of viral SAgs have been identified, such as the nucleocapsid produced by the Rabies virus (Astoul *et al.*, 1996). Furthermore, the fungal species *Candida albicans* and the parasite *Toxoplasma gondii* have been shown to produce proteins with SAg like activity (Devore-Carter *et al.*, 2008, Vallochi *et al.*, 2001). This suggests that Vβ-specific T-cell activation is important in the pathogenesis of diverse microbial infections. Because of their importance in pathogenesis, therapeutic strategies which target SAgs have been the focus of considerable research efforts (Fraser & Proft, 2008). Strategies employing vaccination, passive immunization, peptide antagonists and receptor mimics have

been developed with limited success (Fraser & Proft, 2008). In addition, future therapeutic interventions which target an alternative SAg-specific Lck-independent T-cell signalling pathway have been proposed (Bueno *et al.*, 2006). Importantly, the diversity of SAgs made by different strains and their distinct Vβ dependent binding activities has complicated efforts to design broadly effective therapeutics. Affinity matured forms of Vβ domain derived SAg antagonists, of SEC, TSST-1, and SEB are in development (Buonpane *et al.*, 2005, Buonpane *et al.*, 2007, Kieke *et al.*, 2001). Here we have described the capacity of a single bovine isolate to produce SAgs which activate the entire bovVβ T-cell repertoire, which suggests that a successful Vβ receptor analog would need to be effective against all bovVβ subfamilies. A number of bovine mastitis vaccines currently in development such as MastaVac^{TM} are based on SEC (Chang *et al.*, 2008), however the efficacy of this vaccine may be limited as only 19% of bovine strains examined harbour the *secbov* gene (Smyth *et al*., 2005). In this study, we have identified a novel toxin SElX which is encoded by the great majority of *S. aureus* strains and may represent a novel therapeutic target.

Despite the large number of strains which can produce SAgs, toxinoses such as TSS and food poisoning are a relatively rare outcome, suggesting that such disease symptoms are not the primary role of SAgs in pathogenesis. It has often been proposed that the role of SAgs in pathogenesis is to subvert the normal immune response to the SAg-producing bacteria. The production of SAgs by *S. aureus* may be advantageous in particular niche environments, where competing bacteria are unable to evade the activated immune response (Sriskandan *et al*., 2007). It is feasible that very low concentrations of SAgs may produce local T-cell activation to suppress local inflammation and allow bacterial survival. SAgs have been shown to induce the development of T_{regs} which control the immune response to both self and foreign antigens (Sakaguchi, 2004). Recently, long term exposure to SEC was demonstrated to induce immunosuppressive T_{reg} development at physiologically relevant low concentrations in the bovine immune system (Seo *et al*., 2007, Seo *et al*., 2009). In our future planned experimental IMI it will be important to examine the effect of WT and SAg-deficient strains on the development of CD4+ CD25+ T_{regs} and their role in the persistence of infection. Most humans are exposed to SAgs during their lifetime as has been demonstrated

by the widespread presence of neutralising antibodies (Holtfreter *et al.*, 2006). It has been suggested that inhibition of SAgs by α- and β-globin chains produced in blood prevents TSS (Schlievert *et al.*, 2007). In addition, Chau *et al* have recently proposed that the response to SAg activity is controlled by the bacteria through downregulation of the T-cell response by Toll-like receptor 2 activation (TLR2). This signal initiates the apopotosis pathway for APCs, leads to activation of NF-kβ within the APC, which in turn leads to production of IL-10 inhibiting the response to SAgs (Chau *et al.*, 2009).

In conclusion, the role of SAgs during pathogenesis is very complex. The array of staphylococcal SAgs which have been identified is expanding, and has been expedited with the recent advances in genomics. This diversity is potentially driven by the need to activate a large number of T-cells, and bind to MHC class II molecules in multiple ways, contributing to immune evasion. In this work, we have employed a genome scale approach to investigate SAgs encoded by the bovine isolate RF122. The results of this study contribute to our understanding of staphylococcal SAg diversity and provide a comprehensive analysis of the bovine T-cell response to SAgs. In addition, preliminary experimental infections of dairy cattle with WT and SAg-deficient strains have revealed a potential role for SAgs in the early stages of *S. aureus* infection.

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